# Studies on the characterization of avian influenza viruses isolated from raw poultry products brought to Japan by international flight passengers

（国際旅客により持ち込まれた家きん畜産物から分離された鳥インフルエンザウイルスの性状に関する研究）

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# Contents

# Abbreviations

| | |
|---|---|
| AIV(s): | avian influenza virus(es) |
| AQS: | Animal Quarantine Service |
| BSL: | biosafety level |
| DDBJ: | DNA Data Bank of Japan |
| dpi: | days post-inoculation |
| $EID_{50}$: | 50% egg infectious dose |
| EMBL: | European Molecular Biology Laboratory |
| FAO: | Food and Agriculture Organization of the United Nations |
| FITC: | fluorescein isothiocyanate |
| GISAID: | Global Initiative on Sharing All Influenza Data |
| GM: | geometric mean |
| Gs/GD: | A/goose/Guangdong/1/1996 (H5N1) |
| HA: | haemagglutinin |
| HI: | haemagglutination inhibition |
| HPAIV(s): | highly pathogenic avian influenza virus(es) |
| IVPI: | intravenous pathogenicity index |
| LBM: | live bird market |
| LPAIV(s): | low pathogenic avian influenza virus(es) |
| Mab: | monoclonal antibody |
| MDCK: | Madin-Darby canine kidney |
| MEM: | minimum essential medium |
| MP: | matrix protein |
| NA: | neuraminidase |
| NI: | neuraminidase inhibition |
| NP: | nucleoprotein |
| NS: | nonstructural |
| OIE: | Office international des epizooties (World Organization for Animal Health) |
| PA: | polymerase acidic |
| PB1: | polymerase basic protein 1 |
| PB2: | polymerase basic protein 2 |
| PBS: | phosphate-buffered saline |
| $TCID_{50}$: | 50% tissue culture infectious dose |
| WHO: | World Health Organization |
| YRD: | Yangtze River Delta |

## Notes

Contents of the present thesis were published in the following articles

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2. Shibata A., Okamatsu M., Sumiyoshi R., Matsuno K., Wang Z., Kida H., Osaka H. and Sakoda Y., 2018: Repeated detection of H7N9 avian influenza viruses in raw poultry meat illegally brought to Japan by international flight passengers. *Virology,* 524: 10-17.

3. Shibata A., Harada R., Okamatsu M., Matsuno K., Arita T., Suzuki Y., Shirakura M., Odagiri T., Takemae N., Uchida Y., Saito T., Sakoda Y., Osaka H., 2019: Characterization of a novel reassortant H7N3 highly pathogenic avian influenza virus isolated from a poultry meat product taken on a passenger flight to Japan. *J. Vet. Med. Sci.,* 81: 444-448.

# Preface

Influenza A viruses are negative-sense, single-stranded RNA viruses belonging to the genus *Influenzavirus* A of the family *Orthomyxoviridae*. They have eight viral RNA segments that code for at least 10 different viral proteins: haemagglutinin (HA) protein, neuraminidase (NA) protein, matrix protein (MP), membrane-boundion channel-like protein, nucleoprotein (NP), nonstructural (NS) protein 1, NS protein 2, and three proteins associated with polymerase activity [polymerase acidic (PA), polymerase basic protein 1 (PB1), and polymerase basic protein 2 (PB2)] (1). Influenza A viruses can be classified into subtypes based on antigenicity of their HA and NA, the major surface glycoproteins on virus envelope. There are 16 subtypes of HA (H1-16) and 9 subtypes of NA (N1-9) in influenza A viruses found in birds and mammalian animals (2-4). The subtype diversity of influenza A viruses recently expanded with identification of H17N10 and H18N11, however they are found only in bats (5-6). Wild waterfowls in which all subtypes of HA and NA are found are thought to be natural reservoir hosts of the influenza A viruses (1, 7).

Influenza A viruses originating from birds are called avian influenza viruses (AIVs) which are categorized in highly pathogenic avian influenza viruses (HPAIVs) and low pathogenic avian influenza viruses (LPAIVs) based on their virulence in chickens (8). HPAIVs possess a motif of multiple basic amino acid residues at the HA cleavage site. This cleavage motif is cleaved by ubiquitous cellular proteases such as furin, leading to a systemic infection with severe disease and high mortality in chickens (9, 10). On the other hand, the HA cleavage site of LPAIVs is mono-basic and is cleaved by proteases localized in specific organs or tissues. HPAI outbreaks in poultry are caused by H5 or H7 subtype viruses which are thought to evolve from low pathogenic precursors by introducing some mutations in gene segments and acquiring multiple basic amino acids at the HA cleavage site while circulating in chickens (11-13). When HPAIVs are transmitted back to wild birds, they could be carried long-distance, causing further damage to the poultry industries.

While the migration of wild birds is a major factor in the spread of AIVs over long distance, poultry products also contribute to the spread of AIVs long distance when they are contaminated. In 2007, commercial deep-frozen duck carcasses contaminated with HPAIVs caused suspected incursion into the food chain in Germany, probably associated with following HPAI outbreaks in geographically distant chicken farms (14). In the early 2000s, H5N1 HPAIV has been isolated from duck meat imported from China to South Korea (15) and from China to Japan (16), and low pathogenic H9N2 AIVs have been isolated from chicken meat imported from China to Japan (17, 18).

These contaminated meat products were imported legally for commercial use under control. To import poultry products, a health certificate issued by the exporting country based on the “animal health requirement” is essential. As poultry products from countries with HPAI outbreaks are not allowed to be exported basically, these requirements prevent AIV incursion across the borders.

On the other hand, unlicensed movement of poultry products is a risk for international AIV spread. In 2005, a low pathogenic H10N7 AIV has been isolated from one of the smuggled duck carcasses, which was later seized by official controls in warehouses in Italy (19). However, when or how these carcasses were illegally imported could not be determined. It is not unusual for animal products to be imported illegally. Illegal importation in baggage by international passengers occurs at any airports and ports, making it difficult to control. In fact, many animal products have been illegally imported to Japan as baggage and detected via quarantine inspections at airports and ports. Lately, the number of detected illegal animal products has been increasing along with the growing number of overseas visitors. According to the Animal Quarantine Service (AQS), Ministry of Agriculture, Forestry and Fisheries of Japan, the number of detections of prohibited animal products brought by passengers was about 33,000 cases (45,000 kg) in 2010 and about 94,000 cases (109,000 kg) in 2018 (Table 1).

In the present study, the poultry meat products brought by international passengers and confiscated by AQS were investigated whether they were actually contaminated with infectious AIVs or not. In the investigated period from June 2015 to March 2016, eight AIVs, the H1, H5, and H9 subtypes, including HPAIVs were isolated from the confiscated poultry products brought by flight passengers as hand luggage. The results of antigenic and genetic analyses on the isolates were described in Chapter I.

In the continuous surveillance at the point of entry in Japan from May 2016, the novel H7N9 HPAIVs and LPAIV, and an H7N3 HPAIV were isolated from the confiscated poultry products brought by flight passengers. The novel H7N3 HPAIV contained the HA gene derived from the novel H7N9 HPAIVs isolated in China. The novel strain of H7N9 AIV has emerged and isolated from poultry and human in mainland China since March 2013 (20). Although there have not been reported human-to-human transmission unlike in the case of H5N1 subtype (21-23), the novel H7N9 AIVs caused six epidemic waves with 1,568 laboratory-confirmed cases in human as of 16 October 2020 (24). The results of antigenic and genetic analyses on the H7N9 and H7N3 isolates were described in Chapter II.

This is the first report of HPAIVs from raw poultry products illegally brought to international airport by flight passengers from Asian countries. The present study clearly demonstrated the contribution of international flight passengers carrying illegal meat

products to HPAIV spread. These results also help further understanding of the circulation and genetic evolution of AIVs in endemic areas and provide a better understanding of the importance of border control at the international airports.

**Table 1.** Amount of confiscated animal products brought in hand luggage in each year in Japan

| Year | Number of cases | Weight (kg) |
|---|---|---|
| 2010 | 32,560 | 45,144 |
| 2011 | 35,123 | 48,250 |
| 2012 | 39,318 | 52,607 |
| 2013 | 45,292 | 65,021 |
| 2014 | 57,351 | 85,695 |
| 2015 | 62,742 | 83,313 |
| 2016 | 84,025 | 106,351 |
| 2017 | 94,552 | 119,113 |
| 2018 | 93,897 | 109,056 |

The amounts in this table are based on statistical information of AQS.

# Chapter I

## Isolation and characterization of avian influenza viruses from raw poultry products illegally imported to Japan by international flight passengers

# Introduction

HPAIVs of A/goose/Guangdong/1/1996 (H5N1) (Gs/GD) lineage (25), detected in China in 1996, have circulated in poultry worldwide. Since 2003, their descendants have spread and caused outbreaks in poultry in Asia, Europe, Africa, and North America (26). The evolution of H5N1 viruses has led to the emergence of phylogenetic groups classified into clades based on sequence homology of HA genes (27). Lately, viruses of clades 2.3.2.1c and 2.3.4.4 have been prevailing in poultry of East and Southeast Asia and caused outbreaks worldwide. In Japan, sporadic outbreaks caused by clade 2.3.4.4 have occurred in poultry farms during the winter seasons 2014–2015, 2016–2017, and 2017–2018, probably caused by migratory birds (28-30). To predict outbreaks in poultry, a comprehensive monitoring of AIV targeting wild birds is conducted in cooperation with various organizations in Japan.

AIVs could be transported long-distance not only by migratory birds which have been intensively monitored as a risk factor for incursion of HPAIVs, but also by contaminated raw poultry products which contributes to the nationwide and worldwide spread of HPAIVs across the border (14-16). In addition to the live birds, it is important to monitor the contamination of AIVs in raw poultry products confiscated by quarantine at the border.

Along with the increase in the number of foreign visitors brought by the further development of international transportation in recent years, many poultry products including raw carcasses have been brought to international airports and ports in Japan by flight passengers more than ever before. In order to prevent incursions of AIVs through contaminated poultry products, luggage inspections have been conducted under the supervision of AQS at each of the international airports and ports. However, illegal importation of poultry products by flight passengers are never ceasing.

In this Chapter, AIVs, including HPAIVs, were isolated from raw poultry products illegally introduced into Japan by international flight passengers and confiscated at the animal quarantine. Isolates were characterized through genetic and antigenic analyses, and animal experiments were performed to determine the pathogenicity and tissue tropism in the poultry. This is the first report of HPAIVs from raw poultry products illegally imported by flight passengers from Asian countries. The present study should demonstrate that infectious HPAIVs are brought by flight passengers through contaminated poultry products.

## Materials and Methods

***Poultry products confiscated at animal quarantine***

AQS collected 149 specimens comprising of 136 raw poultry products (including organs) and 13 raw poultry eggs (Table 2). Products were confiscated from June 2015 to March 2016 at Tokyo Narita Airport, Tokyo Haneda Airport, Kansai Airport, Chubu Airport, Sendai Airport and Osaka port. Species of poultry samples were identified by visual inspection and sequencing of the mitochondrial DNA as described previously (31).

***Virus isolation***

All 149 confiscated samples shown in Table 2 were used for virus isolation. When poultry products contained organs (lung, liver, kidney, hearts, brain, etc.), they were pooled with meat as one sample. Eggs were smashed and used a whole egg as one sample. Each pooled sample was converted into a 20% homogenate in phosphate-buffered saline (PBS, pH7.4) and centrifuged 10 min at 1,000 × *g* at 4°C. The supernatant was filtered through a sterile 0.45 μm membrane (Millex-HV, Millipore, Bedford, MA, USA), added with equal volume of PBS containing 20,000 U/mL penicillin (Meiji Seika, Tokyo, Japan), 20 mg/mL streptomycin (Meiji Seika) and 15 μg/mL amphotericin B (Bristol-Myers Squibb, Tokyo, Japan), and incubated for 1 h at 15–25°C. The homogenate (0.2 mL) was inoculated into the allantoic cavities of 9- to 11-day-old embryonated chicken eggs and incubated at 35°C for 2–5 days (32). The embryonated chicken eggs were obtained from flocks of healthy and influenza virus antibody-negative chickens. Allantoic fluids were harvested and tested for haemagglutination. Negative allantoic fluids were passaged and tested again using embryonated chicken eggs as described above. For positive samples, subtypes of HA and NA were identified by haemagglutination inhibition (HI) and NA inhibition (NI) tests with antisera to the reference AIV strains. The titre of 50% egg infectious dose ($EID_{50}$) was determined in embryonated chicken eggs using the method of Reed and Muench (33).

***Viruses used in antigenic analysis***

Viruses were grown in 10-day-old embryonated chicken eggs, and infectious allantoic fluids were stored at −80°C until use. A/whooper swan/Hokkaido/1/2008 (Ws/Hok/08) (H5N1) was isolated from a dead whooper swan in Hokkaido, Japan (34). A/mallard/Hokkaido/24/2009 (Mal/Hok/09) (H5N1) was isolated from a faecal sample of a mallard in Hokkaido, Japan (35). A/peregrine falcon/Hong Kong/810/2009

(Pf/HK/09) (H5N1) was kindly provided by Dr. Geraldine, Tai Lung Veterinary Laboratory, Hong Kong Special Administrative Region, China. A/chicken/Yamaguchi/7/2004 (Ck/Yam/04) (H5N1), A/chicken/Ibaraki/1/2005 (Ck/Ibr/05) (H5N2) and A/chicken/Kumamoto/1-7/2014 (Ck/Kum/14) (H5N8) were kindly provided by Dr. Saito, National Institute of Animal Health, Japan (36-38). A/duck/Pennsylvania/10218/1984 (Dk/Pen/84) (H5N2) was kindly provided by Dr. Webster, St. Jude Children's Research Hospital, Tennessee, USA (39).

***Genetic analysis***

All the AIV isolates in this study were sequenced, and three H5 and four H9 isolates were used for phylogenetic analyses. Viral RNA was extracted from the infectious allantoic fluid using QIAamp viral RNA mini Kit (Qiagen, Hilden, Germany). MiSeq libraries were prepared using Next Ultra RNA Library Prep Kit and Multiplex Oligos for Illumina (New England Biolabs, Ipswich, MA, USA) and sequenced on the MiSeq using MiSeq reagent kit v3 (Illumina, San Diego, CA, USA). Reads were *de novo* assembled using CLC Genomic Workbench (CLC bio, Aarhus, Denmark).

Nucleotide sequences were phylogenetically analysed by the maximum-likelihood method with Tamura-Nei model and bootstrap analysis (n = 1,000) using MEGA 7.0 software (40) with default parameters. The sequences of each segment of the H5 and H9 virus isolates obtained in the present study were compared with those of other strains deposited in public databases, GenBank/European Molecular Biology Laboratory (EMBL)/DNA Data Bank of Japan (DDBJ) (https://www.ncbi.nlm.nih.gov/genbank/) and Global Initiative on Sharing All Influenza Data (GISAID) (http://platform.gisaid.org/). The resulting alignments were used for phylogenetic analysis. The gene sequences obtained in the present study have been registered in GenBank/EMBL/DDBJ as shown in Table 3.

***Antigenic analysis***

Antigenic properties of all three H5 isolates were determined to evaluate antigenicity between and within clades via the cross-HI test using chicken (*Gallus gallus*, Julia) polyclonal antisera (41, 42) and fluorescent antibody method with monoclonal antibodies (MAbs) against H5 HA, which exhibited neutralization activity. The AIVs used in the antigenic analysis were selected based on the previous genetic analysis of H5 isolates. Mal/Hok/09 (H5N1) is a non-pathogenic AIV isolated from a faecal sample from migratory ducks, and the antigenicity of this strain is similar to that of other H5 viruses isolated from migratory ducks (35). The antigenicity of Ws/Hok/08

(H5N1) is similar to that of HPAIVs of clade 2.3.2.1 (43). Pf/HK/09 (H5N1) is an HPAIV, and the HA gene of this strain belongs to clade 2.3.4, which is an ancestral clade of 2.3.4.4. Ck/Kum/14 (H5N8) was isolated from a chicken in 2014 at the Kumamoto Prefecture, Japan, and the HA gene of this strain belongs to clade 2.3.4.4. Ck/Yam/04 (H5N1) is an HPAIV, which shows antigenicity of classical HPAIVs. Ck/Ibr/05 (H5N2) is a low pathogenic AIV, and the HA gene of this strain belongs to the North American lineage. HI tests were performed as described previously (32). For the fluorescent antibody assay, Madin-Darby canine kidney (MDCK) cells infected with representative AIV were fixed with cold 100% acetone 8 h post-inoculation. The reactivity patterns of neutralizing MAbs with viruses were investigated via immunofluorescence with a fluorescein isothiocyanate (FITC)-conjugated goat IgG to mouse IgG (ICN Biomedicals, Inc., Costa Mesa, CA, USA) (44, 45). Fluorescence was visualized with an Axiovert 200 inverted microscope (Carl Zeiss MicroImaging, Thornwood, NY, USA).

***Animal experiments***

Two H5 and one H9 representative isolates, namely A/duck/Japan/AQ-HE72/2015 (Dk/HE72) (H5N6), A/chicken/Japan/AQ-HE79/2015 (Ck/HE79) (H5N1) and A/duck/Japan/AQ-HE5/2015 (Dk/HE5) (H9N2), were selected for animal experiments based on the results of the genetic and antigenic characterization. Four-week-old chickens (*Gallus gallus*, Julia) were obtained from Hokkai Starchick, Hokkaido, Japan. Four-week-old domestic ducks (*Anas platyrhynchos var. domesticus,* Cherry Valley) were obtained from Takikawa Shinseien, Hokkaido, Japan. Eight birds were used in each experiment and randomly divided into two groups to compare virus detection or virus titre at 3 days post-inoculation (dpi) and survival rate for 14 days. All chickens and ducks were intranasally inoculated with 100 μL of virus solution containing $10^{6.0}$ $EID_{50}$ of either Dk/HE72 (H5N6), Ck/HE79 (H5N1), or Dk/HE5 (H9N2). At 3 dpi, four birds were euthanized, and the oral and cloacal swabs, blood, brain, trachea, lung, kidney, colon, and breast meat were collected. In case a bird died before being euthanized, swabs, blood, and tissue samples were collected from the bird on the day of death. To prepare a 10% suspension with minimum essential medium (MEM; Nissui Pharmaceutical, Tokyo, Japan), tissue samples were homogenized using a Multi-Beads Shocker (Yasui Kikai, Osaka, Japan). The infectivity titres for swabs, blood, and tissue samples were calculated via 50% tissue culture infectious dose ($TCID_{50}$) using MDCK cells. The other four birds were observed clinically until 14 dpi. All infected animals were kept in self-contained isolator units (Tokiwa Kagaku, Tokyo,

Japan) at the BSL3 biosafety facility at the Faculty of Veterinary Medicine, Hokkaido University, Japan.

***Ethics statements***

All animal experiments were authorized by the Institutional Animal Care and Use Committee of the Graduate School of Veterinary Medicine, Hokkaido University (approval numbers: 11-0152, 13-0109). All experiments were performed according to the guidelines of the committee.

## Results

### *Isolation of AIVs from illegally imported poultry products*

Eight AIVs were identified from 136 raw poultry carcasses (Table 3), and no virus was identified from 13 raw poultry eggs. Viruses including one H5N1, two H5N6, four H9N2 and one H1N2 subtype were isolated from meat and organ-pooled samples of chicken (*Gallus gallus*) and duck (*Anas platyrhynchos* and *Cairina moschata*) carcasses imported from China (Xiamen, Hong Kong, and Shanghai) and Taiwan (Taipei). Virus titres of each pooled sample ranged from $\leq 10^{2.5}$ (viruses were isolated, but the titre was not greater than the calculation limit of $EID_{50}$) to $10^{3.5}$ $EID_{50}$/g.

### *Genetic analysis of H5 virus isolates*

Nucleotide sequences of HA genes of H5 AIVs were phylogenetically divided into two lineages: Eurasian and North American. The H5 viruses in the Eurasian lineage were identified based on the nomenclature defined by WHO/OIE/FAO H5N1 Evolution Working Group (27). The HA gene of Ck/HE79 (H5N1) was classified into clade 2.3.2.1c and was closely related to A/chicken/Wuhan/HAQL07/2014 (H5N1) virus, isolated in a live bird market (LBM) in China (Figure 1). HA and NA genes of Ck/HE79 (H5N1) shared 98.5% and 98.9% nucleotide identity with A/chicken/Wuhan/HAQL07/2014 (H5N1), respectively. Internal gene segments of Ck/HE79 (H5N1) were also genetically close to those of A/chicken/Wuhan/HAQL07/2014 (H5N1) (Figure 2). HA genes of Dk/HE72 (H5N6) and A/chicken/Japan/AQ-HE144/2015 (Ck/HE144) (H5N6) were classified into group C in clade 2.3.4.4 and were closely related to A/duck/Guangdong/GD01/2014 (H5N6). Group C contains H5N6 viruses identified from China and Laos during 2013–2014 and H5N1 viruses identified from China and Vietnam in 2014 (46) as well as several isolates from humans (Figure 1). HA and NA genes of Dk/HE72 (H5N6) shared 98.6% and 99.0% nucleotide identity with A/duck/Guangdong/GD01/2014 (H5N6), respectively, and internal gene segments were genetically close to those of A/duck/Guangdong/GD01/2014 (H5N6) (Figure 2). HA and NA genes of Ck/HE144 (H5N6) shared 98.2% and 98.2% nucleotide identity with A/duck/Guangdong/GD01/2014 (H5N6), respectively. Internal gene segments (except the PB2 and PB1) of Ck/HE144 (H5N6) were genetically close to those of A/duck/Guangdong/GD01/2014 (H5N6) (Figure 2). PB2 of Ck/HE144 (H5N6) was closely related to that of A/muscovy duck/Vietnam/LBM754/2014 (H5N6), and PB1 of Ck/HE144 (H5N6) was closely related to that of A/duck/Hunan/7/2015 (H3N6), isolated in Hunan Province in South China on the Eastern Asian-Australian Flyway of

migratory birds (47). All H5 isolates contained multiple basic amino acids, arginine (R) and lysine (K), at the proteolytic cleavage site of the HA protein, PQRERRRKR-GLF or PLRERRRKR-GLF, identical to the typical cleavage site motif of H5 HPAIVs (Table 3).

***Genetic analysis of H9 virus isolates***

HA genes of H9 AIVs were phylogenetically divided into two lineages: Eurasian and North American. The Eurasian lineage was further divided into three major sublineages: Y280, G1 and Korean (48). All four H9 isolates were classified as Y280 sublineage of the Eurasian lineage. Three isolates, Dk/HE5 (H9N2), A/duck/Japan/AQ-HE28/2015 (Dk/HE28) (H9N2) and A/chicken/Japan/AQ-HE61/2015 (Ck/HE61) (H9N2), were closely related to the A/chicken/Guangdong/H07/2013 (H9N2) (Figure 3). A/chicken/Japan/AQ-HE14/2015 (Ck/HE14) (H9N2) was closely related to the A/chicken/Guangdong/LZL01/2012 (H9N2). These two related strains were the dominant lineage in Guangdong province in South China in 2012–2013 (49) and classified into another cluster in A/duck/Zhejiang/C1036/2009-like (Dk/ZJ/09-like) strains in the phylogenetic tree (50). HA genes of Dk/HE5 (H9N2), Dk/HE28 (H9N2) and Ck/HE61 (H9N2) shared 98.0%, 98.2% and 98.2% nucleotide identity with A/chicken/Guangdong/H07/2013 (H9N2), respectively, and the HA gene of Ck/HE14 (H9N2) shared 97.6% nucleotide identity with A/chicken/Guangdong/LZL01/2012 (H9N2). NA genes of H9 isolates were classified separately. Internal gene segments showed diversity at the nucleotide level but were closely related to Dk/ZJ/09-like strains (Figure 2). The cleavage site of HA protein of all H9 isolates contained a PSRSSR-GLF motif (Table 3) corresponding to the cleavage site motif of H9N2 viruses in China (49).

***Antigenic analysis of H5 viruses***

H5 isolates were antigenically analysed by cross-HI test and fluorescent antibody assay to reveal antigenic variation of H5 HPAIVs. Chicken anti-serum against the reference virus of clade 2.3.4.4, Ck/Kum/14 (H5N8), effectively inhibited haemagglutination of Dk/HE72 (H5N6) and Ck/HE144 (H5N6). On the contrary, chicken anti-serum against the reference virus of clade 2.3.2.1, Ws/Hok/08 (H5N1), modestly inhibited haemagglutination of Ck/HE79 (H5N1). These viruses exhibited low HI titres in comparison with homologous titres in reactions with antisera of different clades (Table 4).

H5 isolates were antigenically analysed using a panel of neutralizing MAbs recognizing antigenic sites on the HA protein (Table 5). MAbs of Dk/Pen/84 (H5N2) recognize amino acid residue at position 88 (D101/1), 145 (A310/39), 157 (64/1), 168

(B220/1) and 205 (25/2) based on the H3 numbering system (45). MAbs of CK/Kum/14 (H5N8) recognize amino acid residue at position 160 (A32/2), 50 (A262/2), 47 and 287 (B3/2) and 124 (B157/1) based on the H3 numbering system (44). Each MAb of Dk/Pen/84 (H5N2) bound low pathogenic AIV, Mal/Hok/09 (H5N1), while most of the MAbs did not bind Gs/GD-like HPAIVs, including H5 isolates. On the contrary, all MAbs of Ck/Kum/14 (H5N8) bound Dk/HE72 (H5N6), Ck/HE144 (H5N6) and A/chicken/Miyazaki/7/2014 (H5N8) classified in the same genetic clade as Ck/Kum/14 (H5N8). However, the reactivity of MAbs of Ck/Kum/14 (H5N8) to H5 viruses classified in different clades was different: three out of four MAbs reacted with Ck/HE79 (H5N1); two out of four MAbs reacted with Ws/Hok/08 (H5N1) and Pf/HK/09 (H5N1) (Table 5). From the results of the cross-HI test using anti-serum to Ws/Hok/08 and the fluorescent antibody assay using A32/2 MAb, H5N1 isolate of clade 2.3.2.1c further drifted from previously known viruses of clade 2.3.2.1. On the other hand, H5N6 isolates of clade 2.3.4.4 had the same reactivity patterns as previously known viruses of clade 2.3.4.4.

***Pathogenicity of H5 and H9 representative isolates in chickens***

To examine the pathogenicity of H5 and H9 representative isolates in chickens, Dk/HE72 (H5N6), Ck/HE79 (H5N1) and Dk/HE5 (H9N2) were intranasally inoculated into eight chickens. Infected chickens were divided into two groups: one group of four chickens was euthanized at 3 dpi for collecting swabs, blood and tissue samples to determine virus detection, while the other group of four chickens was observed for clinical symptoms and survival for 14 days. In the former group, two chickens inoculated with Ck/HE79 (H5N1) and all chickens inoculated with Dk/HE72 (H5N6) died within 3 dpi, and swabs, blood and tissue samples from dead chickens were taken at the day of death. The remaining chickens were euthanized, and samples were taken at 3 dpi. Virus titres of collected samples were determined (Table 6). Viruses were recovered from each tissue sample inoculated with Dk/HE72 (H5N6) and Ck/HE79 (H5N1), indicating that these viruses caused systemic infection in chickens. No noticeable difference in virus detection in each tissue was observed between Dk/HE72 (H5N6) and Ck/HE79 (H5N1). Virus titres from skeletal muscles, ranging from $10^{4.6}$ to $10^{6.3}$ $TCID_{50}$/g ($10^{5.7}$ $TCID_{50}$/g of the geometric mean (GM) value) of Dk/HE72 (H5N6) and from $10^{3.3}$ to $10^{7.0}$ $EID_{50}$/g ($10^{5.3}$ $TCID_{50}$/g of the GM value) of Ck/HE79 (H5N1), were practically equal to those of brain, trachea, lungs, kidneys and colon. Low titres of virus were recovered from the respiratory tract of chickens inoculated with Dk/HE5 (H9N2). In the latter group, all chickens inoculated with Dk/HE72 (H5N6) and

Ck/HE79 (H5N1) died at 3 dpi, whereas all chickens inoculated with Dk/HE5 (H9N2) survived for 14 days without exhibiting any clinical signs (Figure 4a).

***Pathogenicity of H5 and H9 representative isolates in ducks***

To examine the pathogenicity of H5 and H9 representative isolates in ducks, Dk/HE72 (H5N6), Ck/HE79 (H5N1) and Dk/HE5 (H9N2) were intranasally inoculated into eight domestic ducks. Infected ducks were divided into two groups: one group of four ducks was euthanized at 3 dpi for collecting swabs, blood and tissue samples for virus detection; the other group of four ducks was observed for clinical symptoms and survival for 14 days. In the former group, all ducks were euthanized, and samples were taken at 3 dpi. Virus titres in collected samples were determined (Table 6). In ducks inoculated with Ck/HE79 (H5N1), frequencies of virus detection were high for trachea (4/4), lungs (4/4), kidneys (4/4), colon (4/4), oral swab (4/4) and muscles (3/4), while they were low for cloacal swab (1/4) and blood (0/4). In ducks inoculated with Dk/HE72 (H5N6), frequencies of virus detection were high for trachea (3/4), lungs (3/4), kidneys (3/4) and oral swab (3/4), while they were low for colon (1/4), muscles (1/4), brain (0/4), cloacal swab (0/4) and blood (0/4). Virus was not recovered from any tissues, swabs and blood samples collected from one out of the four ducks inoculated with Dk/HE72 (H5N6). No virus was detected from the respiratory tract of ducks inoculated with Dk/HE5 (H9N2). In the latter group, all ducks inoculated with Dk/HE72 (H5N6), Ck/HE79 (H5N1) and Dk/HE5 (H9N2) survived for 14 days without exhibiting any clinical signs (Figure 4b).

## Discussion

In the present study, eight AIVs including three HPAIVs were isolated from poultry products illegally imported to Japan by international flight passengers. The results suggested that contaminated meat products played a role in carrying infectious HPAIV abroad. Most of the poultry meat products from which AIVs were isolated were imported in disposable plastic bags, indicating their association with the origin of meat products (LBM or backyard slaughtering). On the contrary, the poultry meat product from which Ck/HE14 (H9N2) was isolated was packed in an airtight bag with a commercial logo, suggesting that it may be distributed in the cold chain and underwent a supply chain with controlled temperature to preserve virus activity. In fact, the virus titre of this meat product was $10^{3.5}$ $EID_{50}$/g, which was the highest titre among those of the meat products analysed in this study. This indicated that, when infected poultry products entered the cold chain, they were distributed keeping AIV highly active for a long period. The survival of AIV during long-distance transportation led to the spread of the virus, as reported in Germany in 2007 (14).

The muscle tissue tropism of AIVs is important in spreading viruses by meat products (15), and the level of virus detected in skeletal muscles varies with HPAIV strain (51, 52). GM values of the virus titre of chicken skeletal muscles inoculated with Dk/HE72 (H5N6) and Ck/HE79 (H5N1) were $10^{5.7}$ $TCID_{50}$/g and $10^{5.3}$ $TCID_{50}$/g, respectively. These values were almost the same as those of chicken breast tissue (ranging from $10^{5.3}$ to $10^{5.5}$ $EID_{50}$/g) inoculated with A/duck/Anyang/AVL-1/2001 (H5N1) isolated from duck meat imported from China to South Korea in 2001 (15). Dk/HE72 (H5N6) and Ck/HE79 (H5N1) were also detected from skeletal muscles of some experimentally infected ducks, although their virus titres were below the detection limit or lower than those in the trachea, lungs, and kidney tissues. The tissue tropisms of these isolates in ducks were similar to that of A/duck/Anyang/AVL-1/2001 (H5N1) as previously reported (15). Some HPAIVs replicate systemically and cause mortality; however, ducks are often clinically healthy or mildly diseased when infected with HPAIVs (18, 53). Factors influencing the clinical course of HPAIV-infected ducks depend on species, breeds, age and virus strain, and the presence of HPAIV in skeletal muscles is not related to the ability to cause disease in waterfowl (54, 55). Thus, the absence of clinical signs does not exclude virus detection in muscles (51). In this study, none of the ducks inoculated with either Dk/HE72 (H5N6) or Ck/HE79 (H5N1) showed any clinical signs during the 14-day observation period. This suggests that the risk of contamination of HPAIV into the processed meat of ducks would be higher than that of chickens because of the difficulty of distinguishing asymptomatic infected ducks (54).

Unlike ducks, chickens infected with HPAIV showed clinical symptoms. However, vaccination may lead to overlook HPAIV infection and potentially exacerbate virus spreading by meat product. In fact, vaccination against HPAIV in poultry reduces mortality and morbidity of flocks but does not prevent infection and virus shedding completely (32, 56). Although chickens experimentally inoculated with Ck/HE79 (H5N1) died within 3 dpi, Ck/HE79 (H5N1) was isolated from a chicken product. Diseased birds are usually not used for meat consumption. Thus, it was highly possible that the chicken was apparently healthy although infected with the H5N1 HPAIV. This led us to hypothesize that the chicken was vaccinated, suggesting that the vaccination may play important roles in the incursion of HPAIVs through chicken meat products by hiding clinical signs.

For the low pathogenic AIV isolates, four H9N2 subtypes and one H1N2 subtype were isolated, while no virus was detected from skeletal muscles of chickens and ducks experimentally inoculated with Dk/HE5 (H9N2). A previous study showed that co-infection of an H9N2 virus with bacteria enhanced the virus replication and led to extrapulmonary infection in chickens (18), explaining the isolation of H9N2 virus from poultry meat products in this study. One H1N2 was isolated from pooled suspension of duck meat product, including a part of intestine. It has been reported that H1 subtypes circulated in poultry in South China (57).

For the illegally imported meat products, AQS was able to confirm the flight number of origin, but AQS could not completely trace the origin of poultry products as passengers might transship before the last loading place. Accordingly, the present study indicated the last land in case of transshipping as “the loading country or region” of poultry product. In fact, an H9N2 virus, Ck/HE61 (H9N2), was isolated from a chicken meat product shipped from Taipei, Taiwan; however, there has been no report of H9N2 virus isolation from poultry in Taiwan. The phylogenetic analysis suggested that the HA gene of Ck/HE61 (H9N2) was genetically close to Chinese isolates such as A/chicken/Guangdong/H07/2013 (H9N2) and A/chicken/Wuhan/JXQL01/2015 (H9N2). The chicken meat product might be brought from these regions after being transshipped in Taiwan. The remaining seven strains of H5 and H9 subtypes were directly shipped from South China, consistent with the results of genetic analyses.

The present study showed a strong evidence of infectious HPAIV incursion into Japan through poultry meat products illegally imported by international flight passengers. Since sporadic avian to human infections of H5 HPAIV and H9N2 AIV have been reported, bringing poultry products contaminated with these viruses also has the potential risk of infection to human (58-62). Besides detecting and seizing animal products, it is necessary to publicize and increase awareness to ensure that passengers

do not inadvertently contravene regulations. Thus, quarantine detector dogs as border control measures since 2005 and passenger interviewing since 2011 have been introduced to prevent the spread of transboundary diseases through animal products confiscated at international airports in Japan. However, illegal importation is difficult to control currently more than ever, given the growing number of passengers. To prevent virus incursion through meat products, it is important to continue active efforts as described above. However, to solve this problem, it is essential to encourage the phased cleaning based on the disease control and eradication worldwide, and promote the reduction of the risk of contamination into animal products by dealing with disease-free status.

## Summary

The transportation of poultry and related products for international trade contributes to transboundary pathogen spread and disease outbreaks worldwide. To prevent pathogen incursion through poultry products, many countries have regulations about animal health and poultry product quarantine. However, in Japan, animal products have been illegally introduced into the country in baggage and confiscated at the airport. Lately, the number of illegally imported poultry and the incursion risk of transboundary pathogens through poultry products have been increasing. In this chapter, AIVs were isolated from raw poultry products illegally imported to Japan by international passengers. Highly (H5N1 and H5N6) and low (H9N2 and H1N2) pathogenic AIVs were isolated from raw chicken and duck products carried by flight passengers. H5 and H9 isolates were phylogenetically closely related to viruses isolated from poultry in China, and haemagglutinin genes of H5N1 and H5N6 isolates belonging to clades 2.3.2.1c and 2.3.4.4, respectively. Experimental infections of H5 and H9 isolates in chickens and ducks demonstrated pathogenicity and tissue tropism to skeletal muscles. To prevent virus incursion by poultry products, it is important to continue quarantine inspection at the airports or ports. Furthermore it is essential to encourage the phased cleaning based on the disease control and eradication, and promote the reduction of the risk of virus contamination in poultry products.

**Table 2.** Total number of the illegally imported poultry products investigated from June 2015 to March 2016

| Country or region of loading[a] | Meats | | | Eggs | | | Total |
|---|---|---|---|---|---|---|---|
| | Chicken | Duck | Quail | Chicken | Duck | Quail | |
| China | 43 | 57 | 1 | 2 | 1 | - | 104 |
| Philippines | 12 | - | - | 1 | - | - | 13 |
| Vietnam | 6 | 4 | 1 | 1 | 6 | 2 | 20 |
| Korea | 4 | 2 | - | - | - | - | 6 |
| Taiwan | 3 | 1 | - | - | - | - | 4 |
| Myanmar | 1 | - | - | - | - | - | 1 |
| Egypt | 1 | - | - | - | - | - | 1 |
| Total | 70 | 64 | 2 | 4 | 7 | 2 | 149 |

[a] The last country or region of loading in case of transship

-: None

**Table 3.** Avian influenza viruses isolated from poultry meat products

| Land of loading[a] | Species of poultry | Names | Titre of isolated meat product (log $EID_{50}$/g) | Cleavage site of haemagglutinin | Accession numbers |
|---|---|---|---|---|---|
| Xiamen (China) | *Gallus gallus* | A/chicken/Japan/AQ-HE79/2015 (H5N1) | ≤2.5[b] | PQRERRRKR-GLF | LC208484-91 |
| Hong Kong (China) | *Anas platyrhynchos* | A/duck/Japan/AQ-HE72/2015 (H5N6) | ≤2.5 | PLRERRRKR-GLF | LC208516-23 |
| Shanghai (China) | *Gallus gallus* | A/chicken/Japan/AQ-HE144/2015 (H5N6) | ≤2.5 | PLRERRRKR-GLF | LC208492-99 |
| Xiamen (China) | *Cairina moschata* | A/duck/Japan/AQ-HE5/2015 (H9N2) | ≤2.5 | PSRSSR-GLF | LC208500-07 |
| Shanghai (China) | *Gallus gallus* | A/chicken/Japan/AQ-HE14/2015 (H9N2) | 3.5 | PSRSSR-GLF | LC208468-75 |
| Xiamen (China) | *Cairina moschata* | A/duck/Japan/AQ-HE28/2015 (H9N2) | ≤2.8[b] | PSRSSR-GLF | LC208508-15 |
| Taipei (Taiwan) | *Gallus gallus* | A/chicken/Japan/AQ-HE61/2015 (H9N2) | ≤2.5 | PSRSSR-GLF | LC208476-83 |
| Xiamen (China) | *Cairina moschata* | A/duck/Japan/AQ-HE103/2015 (H1N2) | ≤2.5 | PSVQSK-GLF | - |

[a] The last land in case of transship

[b] Viruses were isolated, but the titre is not greater than the calculation limit of $EID_{50}$.

**Table 4.** Antigenic analysis of H5 influenza viruses with antisera by using haemagglutination inhibition (HI) test

| Genetic lineage | Viruses | Clade | Antiserum to | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Mal/Hok/09 | Ws/Hok/08 | Pf/HK/09 | Ck/Kum/14 | Ck/Yam/04 | Ck/Ibr/05 |
| Eurasia | A/mallard/Hokkaido/24/2009 (H5N1) | - | 1,280 | 80 | 40 | 1,280 | 1,280 | 1,280 |
| | A/whooper swan/Hokkaido/1/2008 (H5N1) | 2.3.2.1 | 40 | 640 | 40 | 640 | 640 | <20 |
| | **A/chicken/Japan/AQ-HE79/2015 (H5N1)** | 2.3.2.1c | 80 | 80 | 160 | <20 | 1,280 | 40 |
| | A/peregrine falcon/Hong Kong/810/2009 (H5N1) | 2.3.4 | <20 | 20 | 2,560 | 20 | 80 | <20 |
| | A/chicken/Kumamoto/1-7/2014 (H5N8) | 2.3.4.4 | 20 | 20 | 320 | 640 | 80 | <20 |
| | **A/duck/Japan/AQ-HE72/2015 (H5N6)** | 2.3.4.4 | <20 | <20 | 640 | 640 | 80 | <20 |
| | **A/chicken/Japan/AQ-HE144/2015 (H5N6)** | 2.3.4.4 | 20 | <20 | 1,280 | 320 | 80 | <20 |
| | A/chicken/Yamaguchi/7/2004 (H5N1) | 2.5 | 320 | 320 | 80 | 80 | 5,120 | 320 |
| North America | A/chicken/Ibaraki/1/2005 (H5N2) | - | 320 | 20 | <20 | <20 | 1,280 | 20,480 |

Viruses isolated in this study are highlighted in bold.

Homologous titres are underlined.

-: Virus not belonging to clade 0-9

**Table 5.** Antigenic analysis of H5 influenza viruses with monoclonal antibodies (MAbs)

| Viruses | Clade | MAbs against Ck/Kum/14 (H5N8) | | | | MAbs against Dk/Pen/84 (H5N2) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | A32/2 | A262/2 | B3/2 | B157/1 | D101/1 | A310/39 | 64/1 | B220/1 | 25/2/5 |
| A/mallard/Hokkaido/24/2009 (H5N1) | - | + | + | - | - | + | + | + | + | + |
| A/whooper swan/Hokkaido/1/2008 (H5N1) | 2.3.2.1 | - | + | + | - | + | - | - | - | - |
| **A/chicken/Japan/AQ-HE79/2015 (H5N1)** | **2.3.2.1c** | **+** | **+** | **+** | **-** | **+** | **-** | **-** | **-** | **-** |
| A/peregrine falcon/Hong Kong/810/2009 (H5N1) | 2.3.4 | - | + | + | - | + | - | - | - | - |
| A/chicken/Kumamoto/1-7/2014 (H5N8) | 2.3.4.4 | + | + | + | + | - | - | - | - | - |
| A/chicken/Miyazaki/7/2014 (H5N8) | 2.3.4.4 | + | + | + | + | - | - | - | - | - |
| **A/duck/Japan/AQ-HE72/2015 (H5N6)** | **2.3.4.4** | **+** | **+** | **+** | **+** | **-** | **-** | **-** | **-** | **-** |
| **A/chicken/Japan/AQ-HE144/2015 (H5N6)** | **2.3.4.4** | **+** | **+** | **+** | **+** | **-** | **-** | **-** | **-** | **-** |

Viruses isolated in this study are highlighted in grey.

-: Virus not belonging to clade 0-9

**Table 6.** Virus recovery from chickens and domestic ducks inoculated with each virus at 3 days post-inoculation (dpi)

| Species | Viruses | Sampling day | Swabs (log $TCID_{50}$/ml) | | Blood (log $TCID_{50}$/ml) | Tissue samples (log $TCID_{50}$/g) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Oral | Cloacal | | Brain | Trachea | Lungs | Kidneys | Colon | Muscle |
| Chickens | Dk/HE72 (H5N6) | 3† | 5.0 | ≤1.0 | 6.8 | 6.0 | 7.3 | 8.0 | 6.6 | 5.8 | 5.8 |
| | | 3† | 4.8 | - | 4.8 | 4.6 | 5.3 | 5.8 | 4.8 | 4.8 | 4.6 |
| | | 3† | 5.5 | - | 5.8 | 5.6 | 6.5 | 7.0 | 5.8 | 6.3 | 6.3 |
| | | 3† | 4.0 | - | 4.8 | 5.8 | 5.8 | 6.8 | 5.8 | 4.8 | 6.1 |
| | Ck/HE79 (H5N1) | 3† | 3.8 | 3.3 | 5.8 | 6.8 | 7.0 | 8.1 | 6.8 | 6.3 | 6.3 |
| | | 3 | ≤1.8 | - | - | 4.3 | 3.8 | 4.6 | 4.6 | 3.8 | 3.3 |
| | | 2† | 4.3 | 4.3 | 7.0 | 6.6 | 7.0 | 7.8 | 8.0 | 6.8 | 7.0 |
| | | 3 | 1.8 | - | 4.3 | 4.8 | 4.0 | 4.6 | 5.8 | 5.6 | 4.8 |
| | Dk/HE5 (H9N2) | 3 | 1.8 | - | - | - | 3.8 | - | - | - | - |
| | | 3 | 2.0 | - | - | - | ≤2.0 | - | - | - | - |
| | | 3 | ≤1.0 | - | - | - | 2.8 | - | - | - | - |
| | | 3 | - | - | - | - | 2.8 | - | - | - | - |
| Ducks | Dk/HE72 (H5N6) | 3 | - | - | - | - | - | - | - | - | - |
| | | 3 | 1.8 | - | - | - | 5.6 | 5.3 | 5.0 | - | - |
| | | 3 | 4.0 | - | - | - | 6.6 | 4.8 | 4.3 | ≤2.3 | ≤2.0 |
| | | 3 | 4.3 | - | - | - | 4.8 | 4.3 | 3.6 | - | - |
| | Ck/HE79 (H5N1) | 3 | 4.3 | - | - | 3.3 | 4.0 | 4.6 | 3.3 | 1.3 | ≤3.1 |
| | | 3 | 4.3 | ≤1.3 | - | 4.1 | 3.8 | 6.6 | 5.6 | ≤2.5 | 3.6 |
| | | 3 | 3.3 | - | - | - | 3.8 | 5.0 | 4.3 | 5.6 | ≤2.6 |
| | | 3 | 4.8 | - | - | - | 5.0 | 4.3 | 4.1 | 4.3 | - |
| | Dk/HE5 (H9N2) | 3 | - | - | - | - | - | - | - | - | - |
| | | 3 | - | - | - | - | - | - | - | - | - |
| | | 3 | - | - | - | - | - | - | - | - | - |
| | | 3 | - | - | - | - | - | - | - | - | - |

-: <0.8 (swabs and blood); <1.8 (tissue samples)  †: Day of death

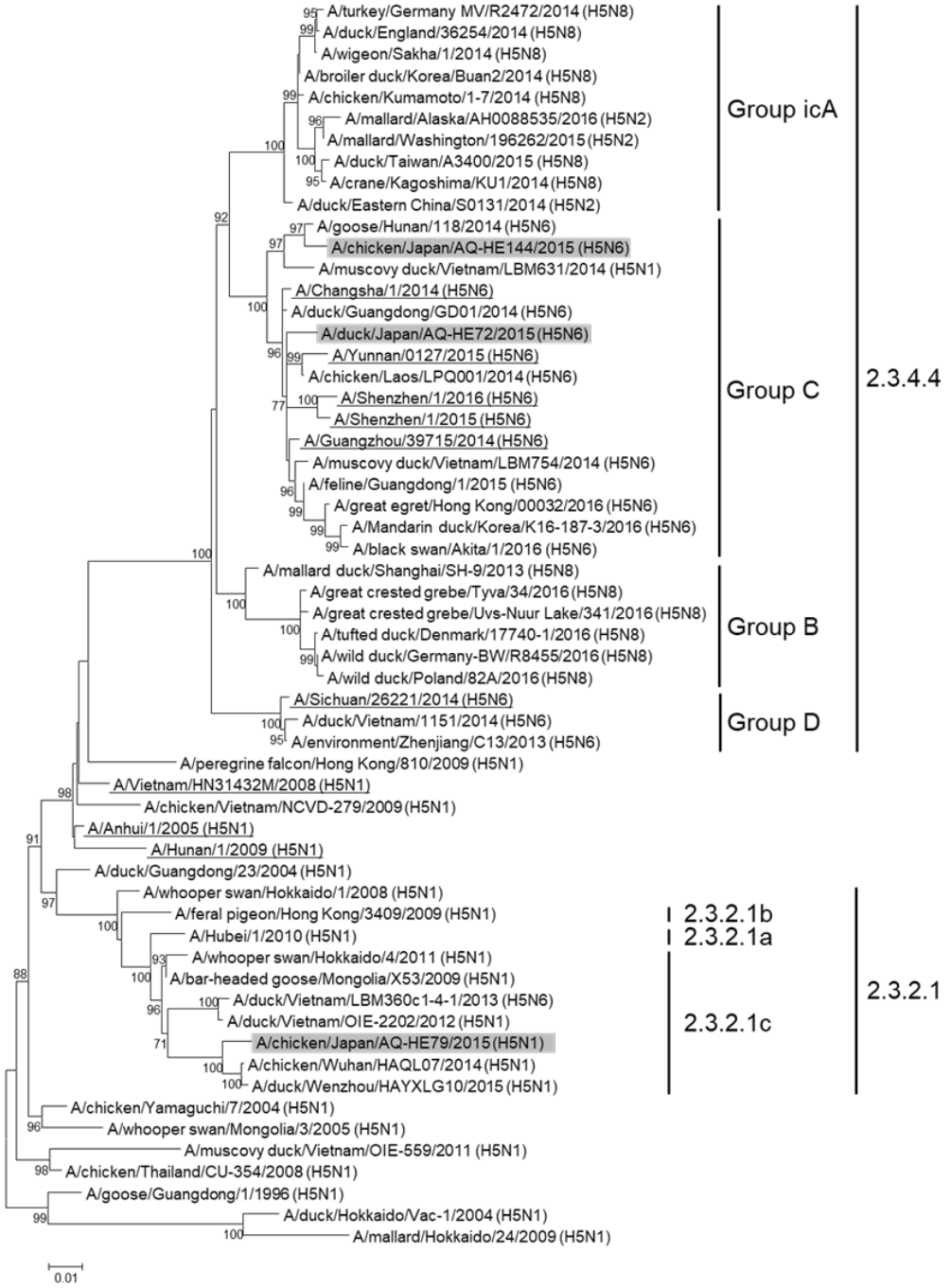



**Figure 1. Phylogenetic tree for the H5 HA genes:** Nucleotides 30-1,733 of the HA gene were analysed using the maximum-likelihood (ML) method with MEGA 7.0 software. Horizontal distances are proportional to the minimum number of nucleotide difference required to join nodes and sequences. Digits at the nodes indicate the probability of confidence levels in a bootstrap analysis with 1,000 replications. Viruses isolated in this study are highlighted in grey. Isolates from humans are shown underlined.

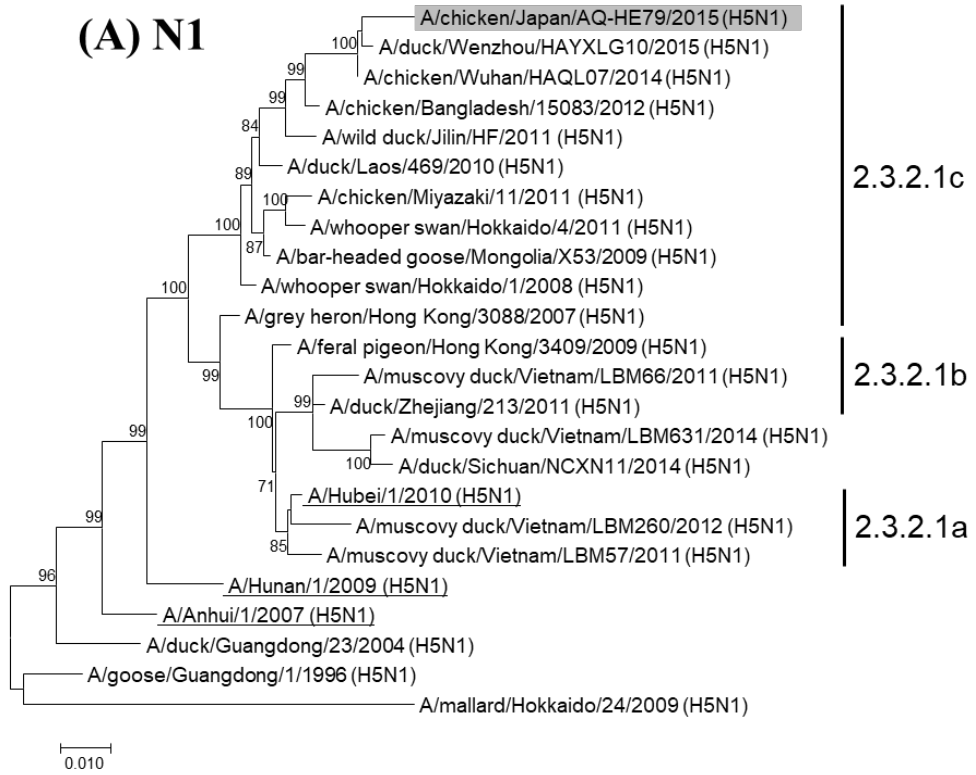
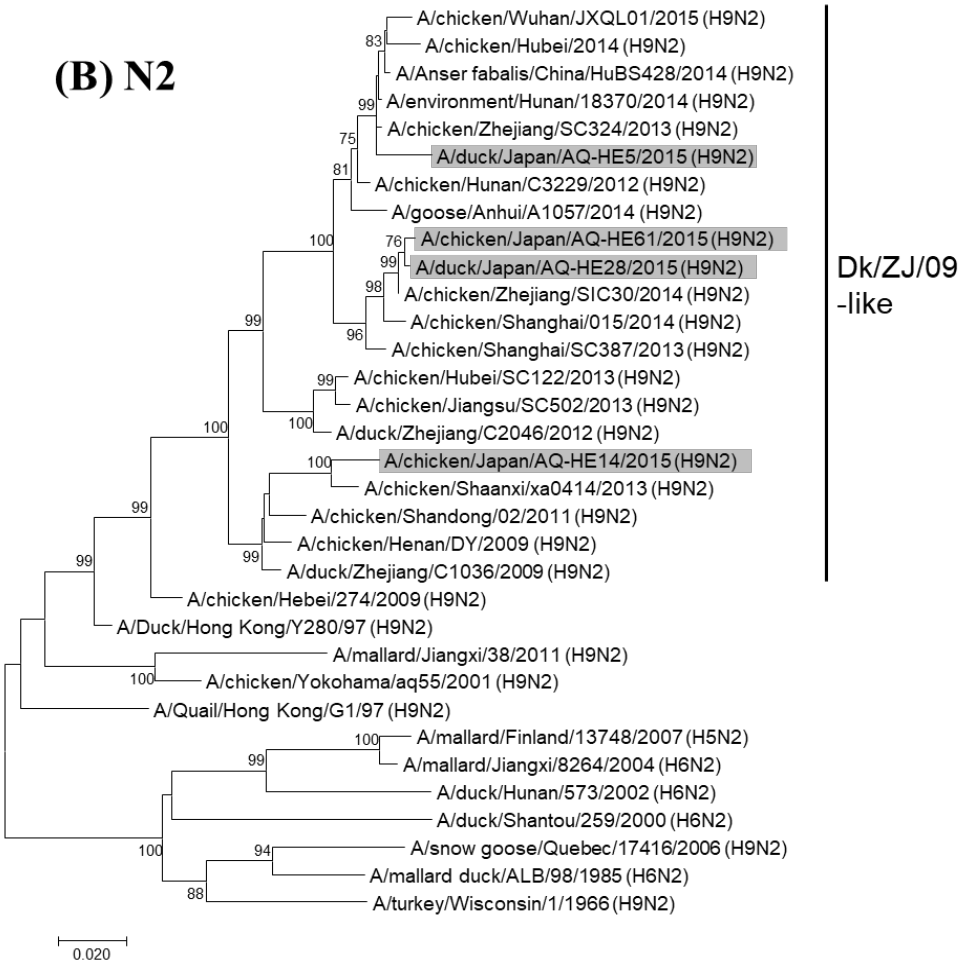
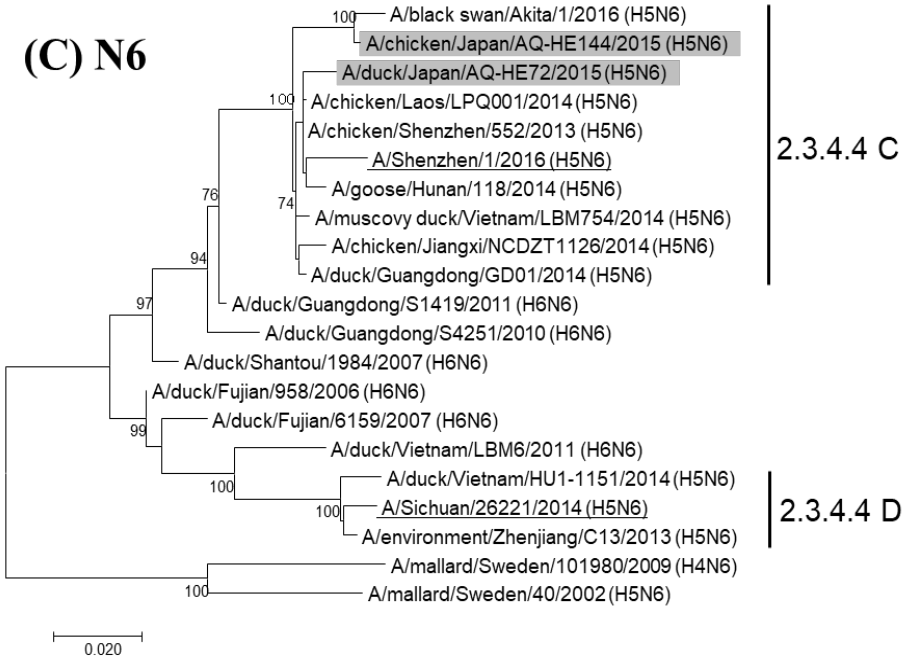
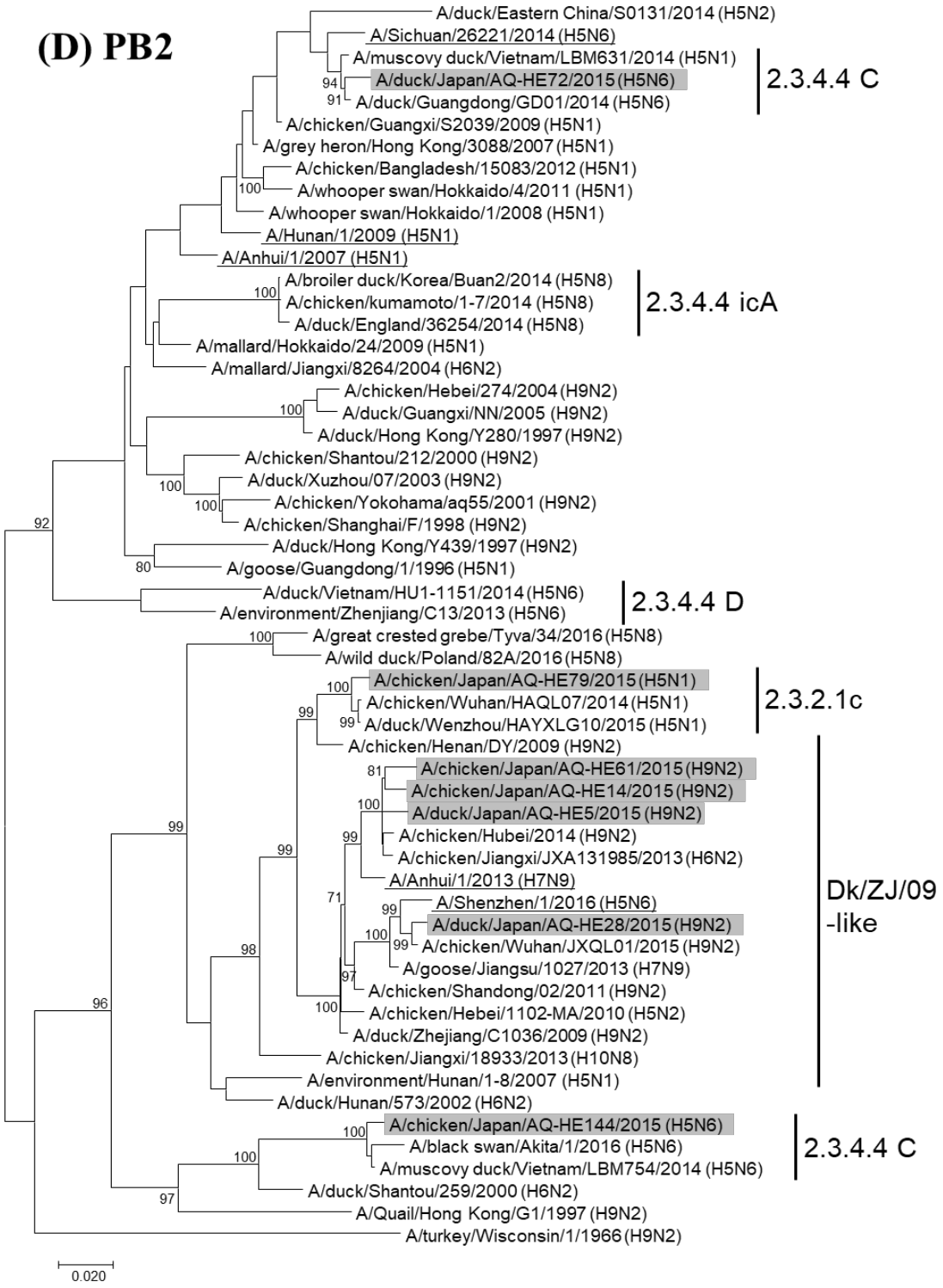



**Figure 2. Phylogenetic tree for the N1NA (A), N2NA (B), N6NA (C), PB2 (D), PB1 (E), PA (F), NP (G), M (H), and NS (I) genes of the isolated viruses.** The trees were analysed using the maximum-likelihood (ML) method with MEGA 7.0 software (Kumar et al., 2016). Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. Digits at the nodes indicate the probability of confidence levels in a bootstrap analysis with 1,000 replications. The viruses isolated in this study are highlighted in grey. The isolates from humans are shown underlined.


(B) N2
A/chicken/Wuhan/JXQL01/2015 (H9N2)
A/chicken/Hubei/2014 (H9N2)
A/Anser fabalis/China/HuBS428/2014 (H9N2)
A/environment/Hunan/18370/2014 (H9N2)
A/chicken/Zhejiang/SC324/2013 (H9N2)
A/duck/Japan/AQ-HE5/2015 (H9N2)
A/chicken/Hunan/C3229/2012 (H9N2)
A/goose/Anhui/A1057/2014 (H9N2)
A/chicken/Japan/AQ-HE61/2015 (H9N2)
A/duck/Japan/AQ-HE28/2015 (H9N2)
A/chicken/Zhejiang/SIC30/2014 (H9N2)
A/chicken/Shanghai/015/2014 (H9N2)
A/chicken/Shanghai/SC387/2013 (H9N2)
A/chicken/Hubei/SC122/2013 (H9N2)
A/chicken/Jiangsu/SC502/2013 (H9N2)
A/duck/Zhejiang/C2046/2012 (H9N2)
A/chicken/Japan/AQ-HE14/2015 (H9N2)
A/chicken/Shaanxi/xa0414/2013 (H9N2)
A/chicken/Shandong/02/2011 (H9N2)
A/chicken/Henan/DY/2009 (H9N2)
A/duck/Zhejiang/C1036/2009 (H9N2)
A/chicken/Hebei/274/2009 (H9N2)
A/Duck/Hong Kong/Y280/97 (H9N2)
A/mallard/Jiangxi/38/2011 (H9N2)
A/chicken/Yokohama/aq55/2001 (H9N2)
A/Quail/Hong Kong/G1/97 (H9N2)
A/mallard/Finland/13748/2007 (H5N2)
A/mallard/Jiangxi/8264/2004 (H6N2)
A/duck/Hunan/573/2002 (H6N2)
A/duck/Shantou/259/2000 (H6N2)
A/snow goose/Quebec/17416/2006 (H9N2)
A/mallard duck/ALB/98/1985 (H6N2)
A/turkey/Wisconsin/1/1966 (H9N2)
0.020
Dk/ZJ/09
-like


(C) N6
A/black swan/Akita/1/2016 (H5N6)
A/chicken/Japan/AQ-HE144/2015 (H5N6)
A/duck/Japan/AQ-HE72/2015 (H5N6)
A/chicken/Laos/LPQ001/2014 (H5N6)
A/chicken/Shenzhen/552/2013 (H5N6)
A/Shenzhen/1/2016 (H5N6)
A/goose/Hunan/118/2014 (H5N6)
A/muscovy duck/Vietnam/LBM754/2014 (H5N6)
A/chicken/Jiangxi/NCDZT1126/2014 (H5N6)
A/duck/Guangdong/GD01/2014 (H5N6)
A/duck/Guangdong/S1419/2011 (H6N6)
A/duck/Guangdong/S4251/2010 (H6N6)
A/duck/Shantou/1984/2007 (H6N6)
A/duck/Fujian/958/2006 (H6N6)
A/duck/Fujian/6159/2007 (H6N6)
A/duck/Vietnam/LBM6/2011 (H6N6)
A/duck/Vietnam/HU1-1151/2014 (H5N6)
A/Sichuan/26221/2014 (H5N6)
A/environment/Zhenjiang/C13/2013 (H5N6)
A/mallard/Sweden/101980/2009 (H4N6)
A/mallard/Sweden/40/2002 (H5N6)
0.020
2.3.4.4 C
2.3.4.4 D


(D) PB2
A/duck/Eastern China/S0131/2014 (H5N2)
A/Sichuan/26221/2014 (H5N6)
A/muscovy duck/Vietnam/LBM631/2014 (H5N1)
A/duck/Japan/AQ-HE72/2015 (H5N6)
A/duck/Guangdong/GD01/2014 (H5N6)
2.3.4.4 C
A/chicken/Guangxi/S2039/2009 (H5N1)
A/grey heron/Hong Kong/3088/2007 (H5N1)
A/chicken/Bangladesh/15083/2012 (H5N1)
A/whooper swan/Hokkaido/4/2011 (H5N1)
A/whooper swan/Hokkaido/1/2008 (H5N1)
A/Hunan/1/2009 (H5N1)
A/Anhui/1/2007 (H5N1)
A/broiler duck/Korea/Buan2/2014 (H5N8)
A/chicken/kumamoto/1-7/2014 (H5N8)
A/duck/England/36254/2014 (H5N8)
2.3.4.4 icA
A/mallard/Hokkaido/24/2009 (H5N1)
A/mallard/Jiangxi/8264/2004 (H6N2)
A/chicken/Hebei/274/2004 (H9N2)
A/duck/Guangxi/NN/2005 (H9N2)
A/duck/Hong Kong/Y280/1997 (H9N2)
A/chicken/Shantou/212/2000 (H9N2)
A/duck/Xuzhou/07/2003 (H9N2)
A/chicken/Yokohama/aq55/2001 (H9N2)
A/chicken/Shanghai/F/1998 (H9N2)
A/duck/Hong Kong/Y439/1997 (H9N2)
A/goose/Guangdong/1/1996 (H5N1)
A/duck/Vietnam/HU1-1151/2014 (H5N6)
A/environment/Zhenjiang/C13/2013 (H5N6)
2.3.4.4 D
A/great crested grebe/Tyva/34/2016 (H5N8)
A/wild duck/Poland/82A/2016 (H5N8)
A/chicken/Japan/AQ-HE79/2015 (H5N1)
A/chicken/Wuhan/HAQL07/2014 (H5N1)
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A/chicken/Hubei/2014 (H9N2)
A/chicken/Jiangxi/JXA131985/2013 (H6N2)
A/Anhui/1/2013 (H7N9)
A/Shenzhen/1/2016 (H5N6)
A/duck/Japan/AQ-HE28/2015 (H9N2)
A/chicken/Wuhan/JXQL01/2015 (H9N2)
A/goose/Jiangsu/1027/2013 (H7N9)
A/chicken/Shandong/02/2011 (H9N2)
A/chicken/Hebei/1102-MA/2010 (H5N2)
A/duck/Zhejiang/C1036/2009 (H9N2)
A/chicken/Jiangxi/18933/2013 (H10N8)
A/environment/Hunan/1-8/2007 (H5N1)
A/duck/Hunan/573/2002 (H6N2)
Dk/ZJ/09
-like
A/chicken/Japan/AQ-HE144/2015 (H5N6)
A/black swan/Akita/1/2016 (H5N6)
A/muscovy duck/Vietnam/LBM754/2014 (H5N6)
2.3.4.4 C
A/duck/Shantou/259/2000 (H6N2)
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A/turkey/Wisconsin/1/1966 (H9N2)
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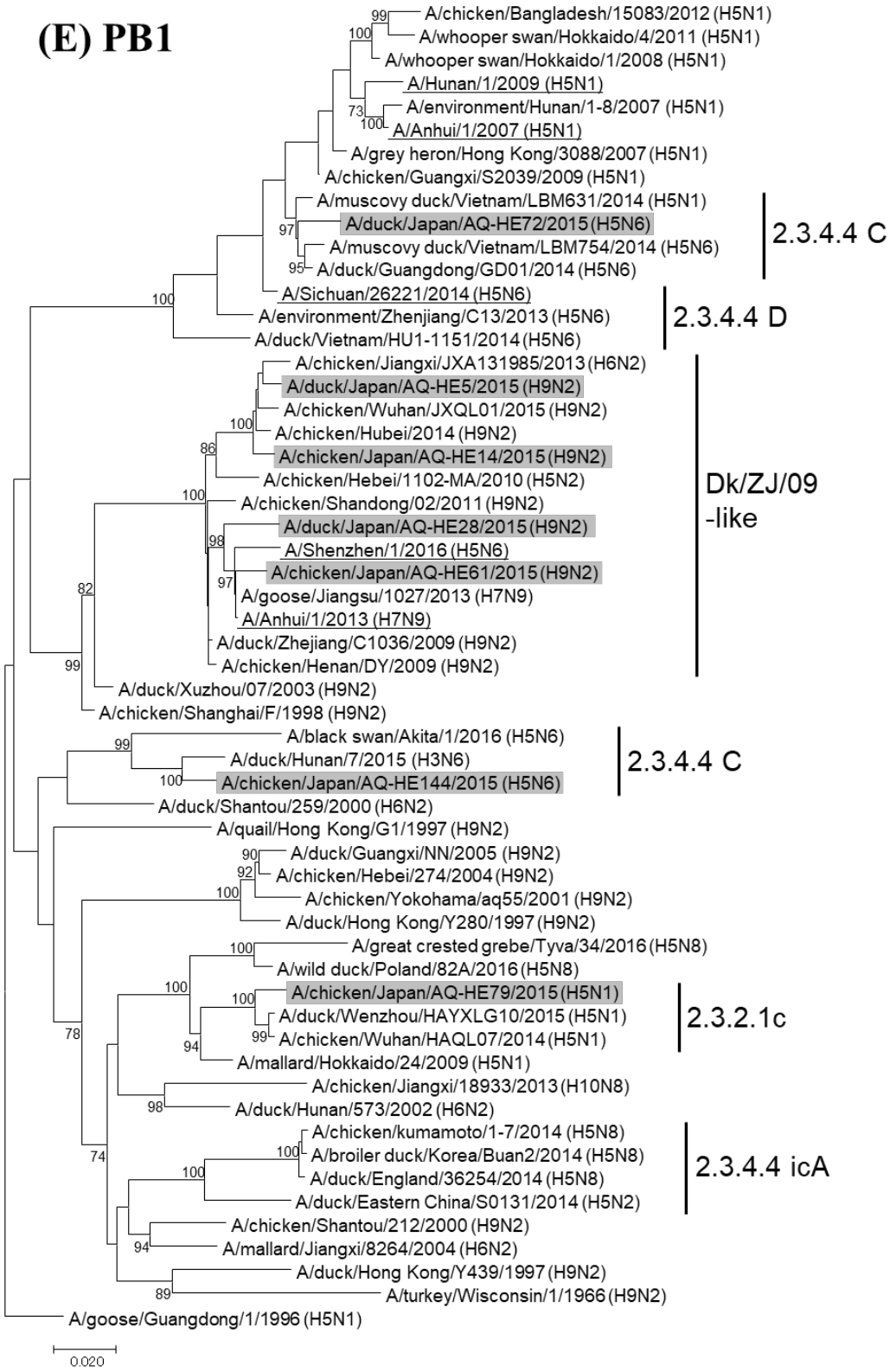
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A/duck/Guangdong/GD01/2014 (H5N6)
2.3.4.4 C
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A/duck/Vietnam/HU1-1151/2014 (H5N6)
2.3.4.4 D
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A/chicken/Wuhan/JXQL01/2015 (H9N2)
A/chicken/Hubei/2014 (H9N2)
A/chicken/Japan/AQ-HE14/2015 (H9N2)
A/chicken/Hebei/1102-MA/2010 (H5N2)
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A/duck/Japan/AQ-HE28/2015 (H9N2)
A/Shenzhen/1/2016 (H5N6)
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A/Anhui/1/2013 (H7N9)
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A/chicken/Henan/DY/2009 (H9N2)
Dk/ZJ/09
-like
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A/chicken/Shanghai/F/1998 (H9N2)
A/black swan/Akita/1/2016 (H5N6)
A/duck/Hunan/7/2015 (H3N6)
A/chicken/Japan/AQ-HE144/2015 (H5N6)
2.3.4.4 C
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A/chicken/Yokohama/aq55/2001 (H9N2)
A/duck/Hong Kong/Y280/1997 (H9N2)
A/great crested grebe/Tyva/34/2016 (H5N8)
A/wild duck/Poland/82A/2016 (H5N8)
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A/duck/Wenzhou/HAYXLG10/2015 (H5N1)
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A/duck/Hunan/573/2002 (H6N2)
A/chicken/kumamoto/1-7/2014 (H5N8)
A/broiler duck/Korea/Buan2/2014 (H5N8)
A/duck/England/36254/2014 (H5N8)
A/duck/Eastern China/S0131/2014 (H5N2)
2.3.4.4 icA
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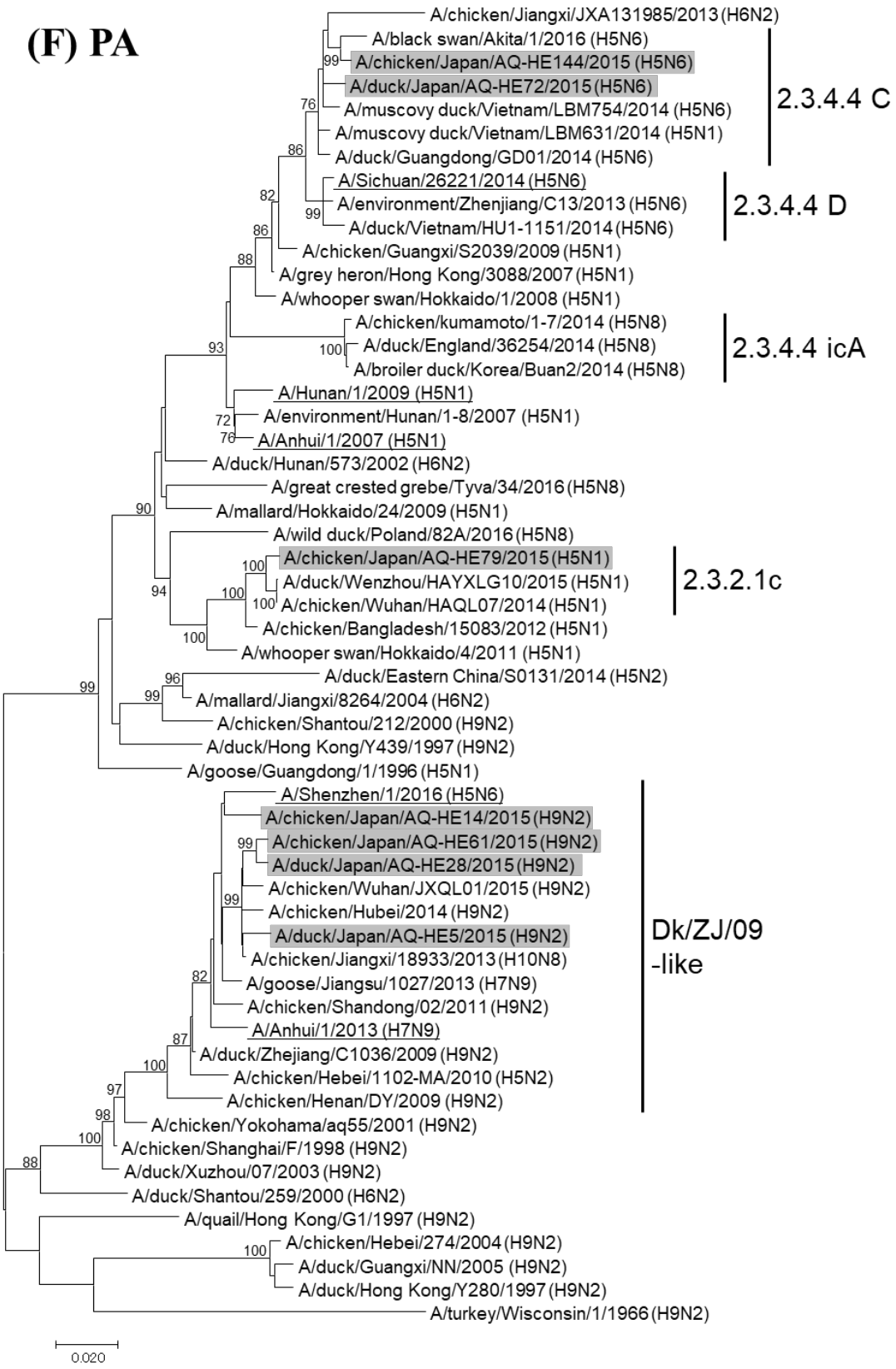
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A/duck/Japan/AQ-HE72/2015 (H5N6)
A/muscovy duck/Vietnam/LBM754/2014 (H5N6)
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A/duck/Guangdong/GD01/2014 (H5N6)
2.3.4.4 C
A/Sichuan/26221/2014 (H5N6)
A/environment/Zhenjiang/C13/2013 (H5N6)
A/duck/Vietnam/HU1-1151/2014 (H5N6)
2.3.4.4 D
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A/whooper swan/Hokkaido/1/2008 (H5N1)
A/chicken/kumamoto/1-7/2014 (H5N8)
A/duck/England/36254/2014 (H5N8)
A/broiler duck/Korea/Buan2/2014 (H5N8)
2.3.4.4 icA
A/Hunan/1/2009 (H5N1)
A/environment/Hunan/1-8/2007 (H5N1)
A/Anhui/1/2007 (H5N1)
A/duck/Hunan/573/2002 (H6N2)
A/great crested grebe/Tyva/34/2016 (H5N8)
A/mallard/Hokkaido/24/2009 (H5N1)
A/wild duck/Poland/82A/2016 (H5N8)
A/chicken/Japan/AQ-HE79/2015 (H5N1)
A/duck/Wenzhou/HAYXLG10/2015 (H5N1)
A/chicken/Wuhan/HAQL07/2014 (H5N1)
2.3.2.1c
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A/whooper swan/Hokkaido/4/2011 (H5N1)
A/duck/Eastern China/S0131/2014 (H5N2)
A/mallard/Jiangxi/8264/2004 (H6N2)
A/chicken/Shantou/212/2000 (H9N2)
A/duck/Hong Kong/Y439/1997 (H9N2)
A/goose/Guangdong/1/1996 (H5N1)
A/Shenzhen/1/2016 (H5N6)
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A/chicken/Japan/AQ-HE61/2015 (H9N2)
A/duck/Japan/AQ-HE28/2015 (H9N2)
A/chicken/Wuhan/JXQL01/2015 (H9N2)
A/chicken/Hubei/2014 (H9N2)
A/duck/Japan/AQ-HE5/2015 (H9N2)
A/chicken/Jiangxi/18933/2013 (H10N8)
A/goose/Jiangsu/1027/2013 (H7N9)
A/chicken/Shandong/02/2011 (H9N2)
A/Anhui/1/2013 (H7N9)
A/duck/Zhejiang/C1036/2009 (H9N2)
A/chicken/Hebei/1102-MA/2010 (H5N2)
A/chicken/Henan/DY/2009 (H9N2)
Dk/ZJ/09
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A/chicken/Shanghai/F/1998 (H9N2)
A/duck/Xuzhou/07/2003 (H9N2)
A/duck/Shantou/259/2000 (H6N2)
A/quail/Hong Kong/G1/1997 (H9N2)
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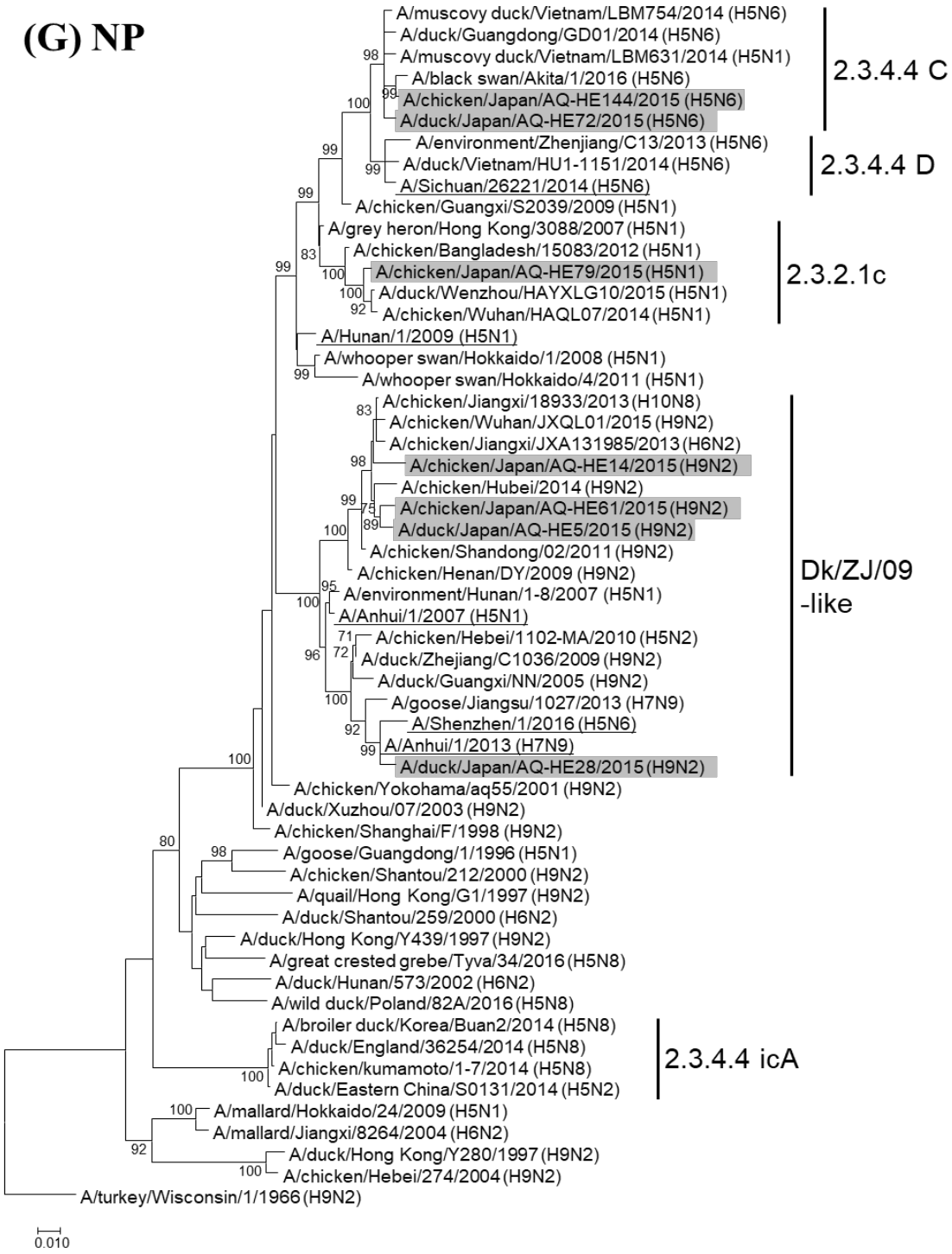
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A/Sichuan/26221/2014 (H5N6)
A/chicken/Guangxi/S2039/2009 (H5N1)
A/grey heron/Hong Kong/3088/2007 (H5N1)
A/chicken/Bangladesh/15083/2012 (H5N1)
A/chicken/Japan/AQ-HE79/2015 (H5N1)
A/duck/Wenzhou/HAYXLG10/2015 (H5N1)
A/chicken/Wuhan/HAQL07/2014 (H5N1)
A/Hunan/1/2009 (H5N1)
A/whooper swan/Hokkaido/1/2008 (H5N1)
A/whooper swan/Hokkaido/4/2011 (H5N1)
A/chicken/Jiangxi/18933/2013 (H10N8)
A/chicken/Wuhan/JXQL01/2015 (H9N2)
A/chicken/Jiangxi/JXA131985/2013 (H6N2)
A/chicken/Japan/AQ-HE14/2015 (H9N2)
A/chicken/Hubei/2014 (H9N2)
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A/duck/Japan/AQ-HE5/2015 (H9N2)
A/chicken/Shandong/02/2011 (H9N2)
A/chicken/Henan/DY/2009 (H9N2)
A/environment/Hunan/1-8/2007 (H5N1)
A/Anhui/1/2007 (H5N1)
A/chicken/Hebei/1102-MA/2010 (H5N2)
A/duck/Zhejiang/C1036/2009 (H9N2)
A/duck/Guangxi/NN/2005 (H9N2)
A/goose/Jiangsu/1027/2013 (H7N9)
A/Shenzhen/1/2016 (H5N6)
A/Anhui/1/2013 (H7N9)
A/duck/Japan/AQ-HE28/2015 (H9N2)
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A/duck/Xuzhou/07/2003 (H9N2)
A/chicken/Shanghai/F/1998 (H9N2)
A/goose/Guangdong/1/1996 (H5N1)
A/chicken/Shantou/212/2000 (H9N2)
A/quail/Hong Kong/G1/1997 (H9N2)
A/duck/Shantou/259/2000 (H6N2)
A/duck/Hong Kong/Y439/1997 (H9N2)
A/great crested grebe/Tyva/34/2016 (H5N8)
A/duck/Hunan/573/2002 (H6N2)
A/wild duck/Poland/82A/2016 (H5N8)
A/broiler duck/Korea/Buan2/2014 (H5N8)
A/duck/England/36254/2014 (H5N8)
A/chicken/kumamoto/1-7/2014 (H5N8)
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A/chicken/Hebei/274/2004 (H9N2)
A/turkey/Wisconsin/1/1966 (H9N2)
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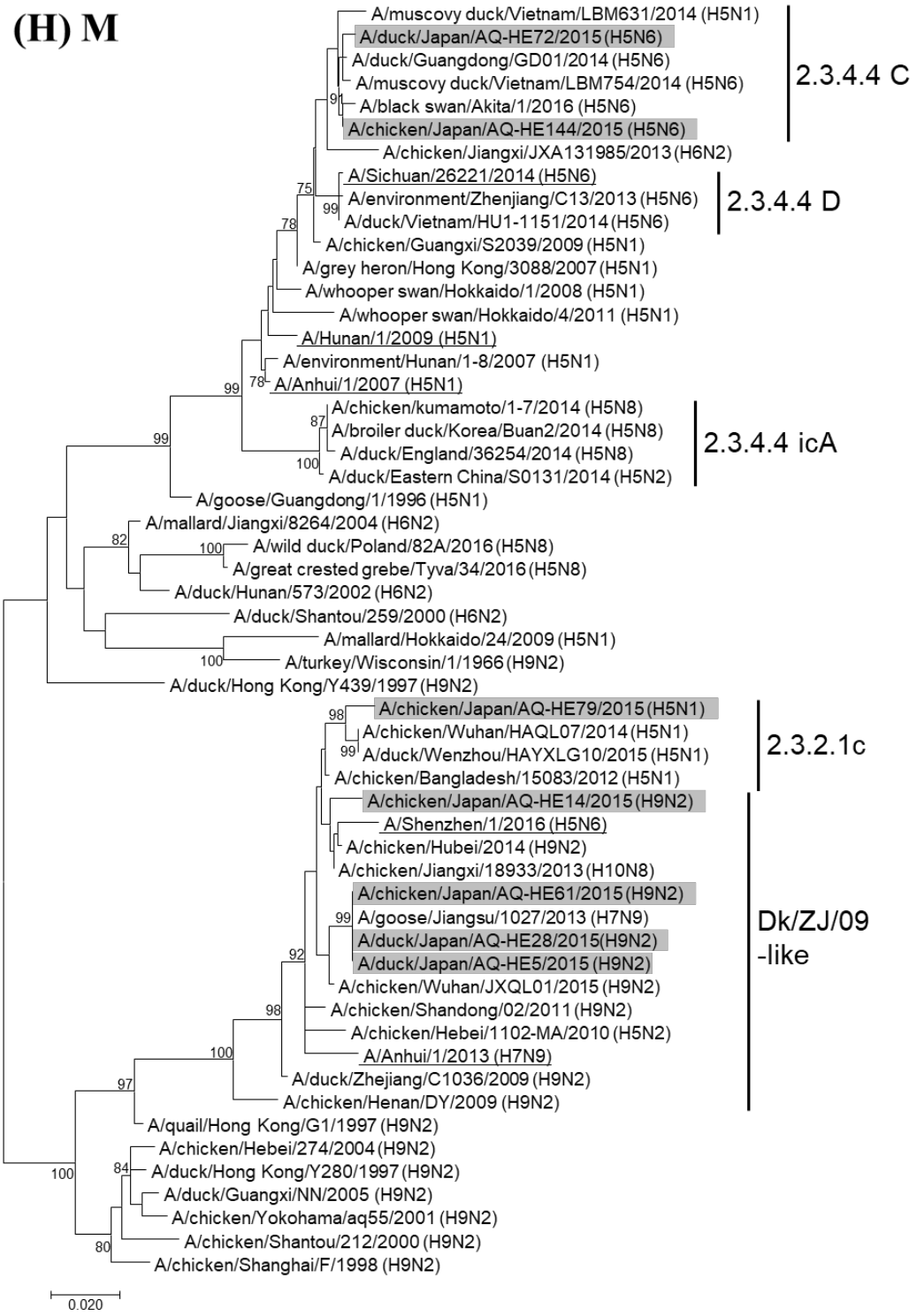
2.3.4.4 D
2.3.2.1c
Dk/ZJ/09
-like
2.3.4.4 icA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A/chicken/Japan/AQ-HE144/2015 (H5N6)
2.3.4.4 C
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A/duck/Vietnam/HU1-1151/2014 (H5N6)
2.3.4.4 D
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A/Anhui/1/2007 (H5N1)
A/chicken/kumamoto/1-7/2014 (H5N8)
A/broiler duck/Korea/Buan2/2014 (H5N8)
A/duck/England/36254/2014 (H5N8)
A/duck/Eastern China/S0131/2014 (H5N2)
2.3.4.4 icA
A/goose/Guangdong/1/1996 (H5N1)
A/mallard/Jiangxi/8264/2004 (H6N2)
A/wild duck/Poland/82A/2016 (H5N8)
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A/turkey/Wisconsin/1/1966 (H9N2)
A/duck/Hong Kong/Y439/1997 (H9N2)
A/chicken/Japan/AQ-HE79/2015 (H5N1)
A/chicken/Wuhan/HAQL07/2014 (H5N1)
A/duck/Wenzhou/HAYXLG10/2015 (H5N1)
A/chicken/Bangladesh/15083/2012 (H5N1)
2.3.2.1c
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A/Shenzhen/1/2016 (H5N6)
A/chicken/Hubei/2014 (H9N2)
A/chicken/Jiangxi/18933/2013 (H10N8)
A/chicken/Japan/AQ-HE61/2015 (H9N2)
A/goose/Jiangsu/1027/2013 (H7N9)
A/duck/Japan/AQ-HE28/2015(H9N2)
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A/Anhui/1/2013 (H7N9)
A/duck/Zhejiang/C1036/2009 (H9N2)
A/chicken/Henan/DY/2009 (H9N2)
Dk/ZJ/09
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A/chicken/Hebei/274/2004 (H9N2)
A/duck/Hong Kong/Y280/1997 (H9N2)
A/duck/Guangxi/NN/2005 (H9N2)
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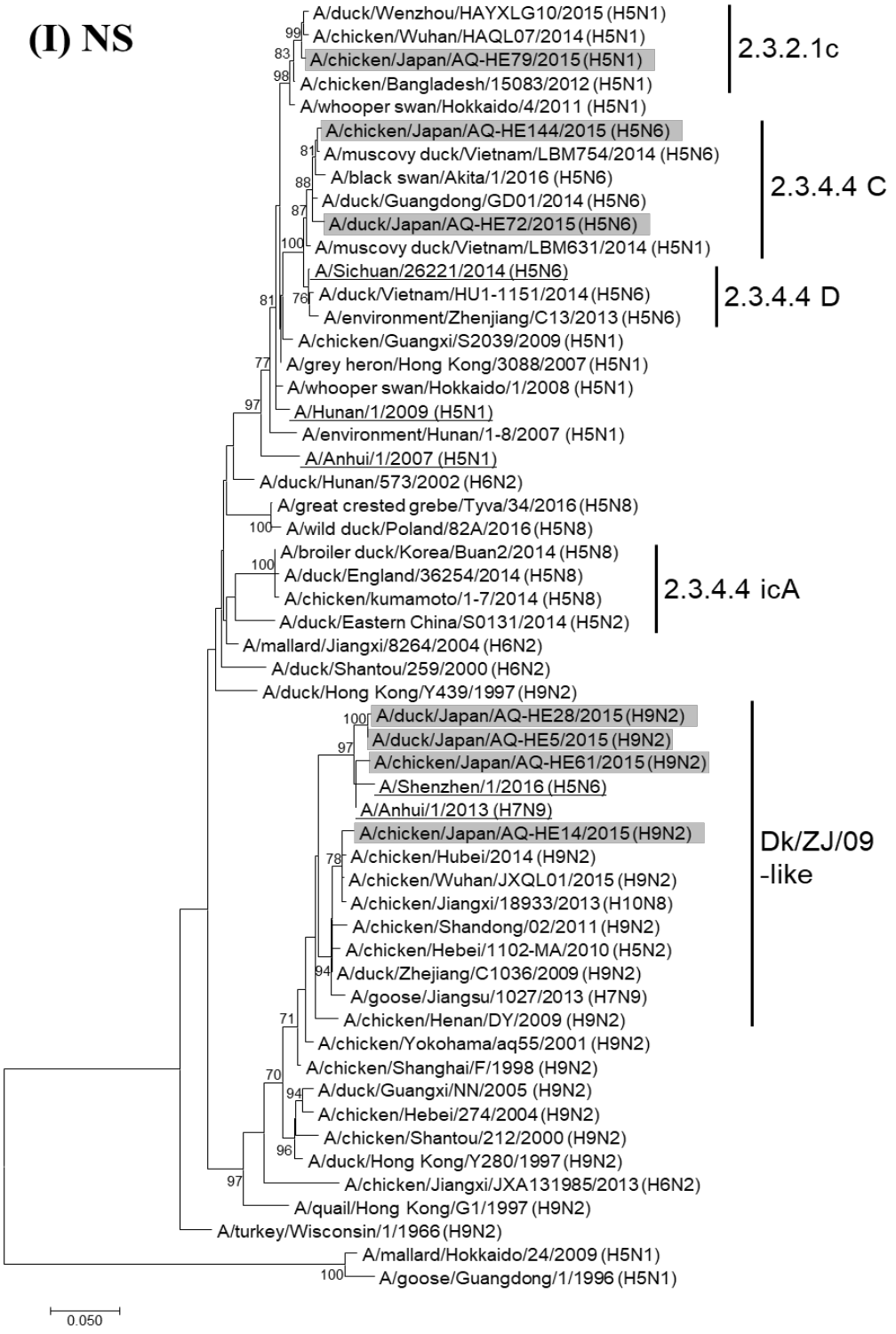
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2.3.2.1c
A/chicken/Japan/AQ-HE144/2015 (H5N6)
A/muscovy duck/Vietnam/LBM754/2014 (H5N6)
A/black swan/Akita/1/2016 (H5N6)
A/duck/Guangdong/GD01/2014 (H5N6)
A/duck/Japan/AQ-HE72/2015 (H5N6)
A/muscovy duck/Vietnam/LBM631/2014 (H5N1)
2.3.4.4 C
A/Sichuan/26221/2014 (H5N6)
A/duck/Vietnam/HU1-1151/2014 (H5N6)
A/environment/Zhenjiang/C13/2013 (H5N6)
2.3.4.4 D
A/chicken/Guangxi/S2039/2009 (H5N1)
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A/whooper swan/Hokkaido/1/2008 (H5N1)
A/Hunan/1/2009 (H5N1)
A/environment/Hunan/1-8/2007 (H5N1)
A/Anhui/1/2007 (H5N1)
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A/great crested grebe/Tyva/34/2016 (H5N8)
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2.3.4.4 icA
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A/duck/Shantou/259/2000 (H6N2)
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A/duck/Japan/AQ-HE5/2015 (H9N2)
A/chicken/Japan/AQ-HE61/2015 (H9N2)
A/Shenzhen/1/2016 (H5N6)
A/Anhui/1/2013 (H7N9)
A/chicken/Japan/AQ-HE14/2015 (H9N2)
A/chicken/Hubei/2014 (H9N2)
A/chicken/Wuhan/JXQL01/2015 (H9N2)
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A/turkey/Wisconsin/1/1966 (H9N2)
Dk/ZJ/09
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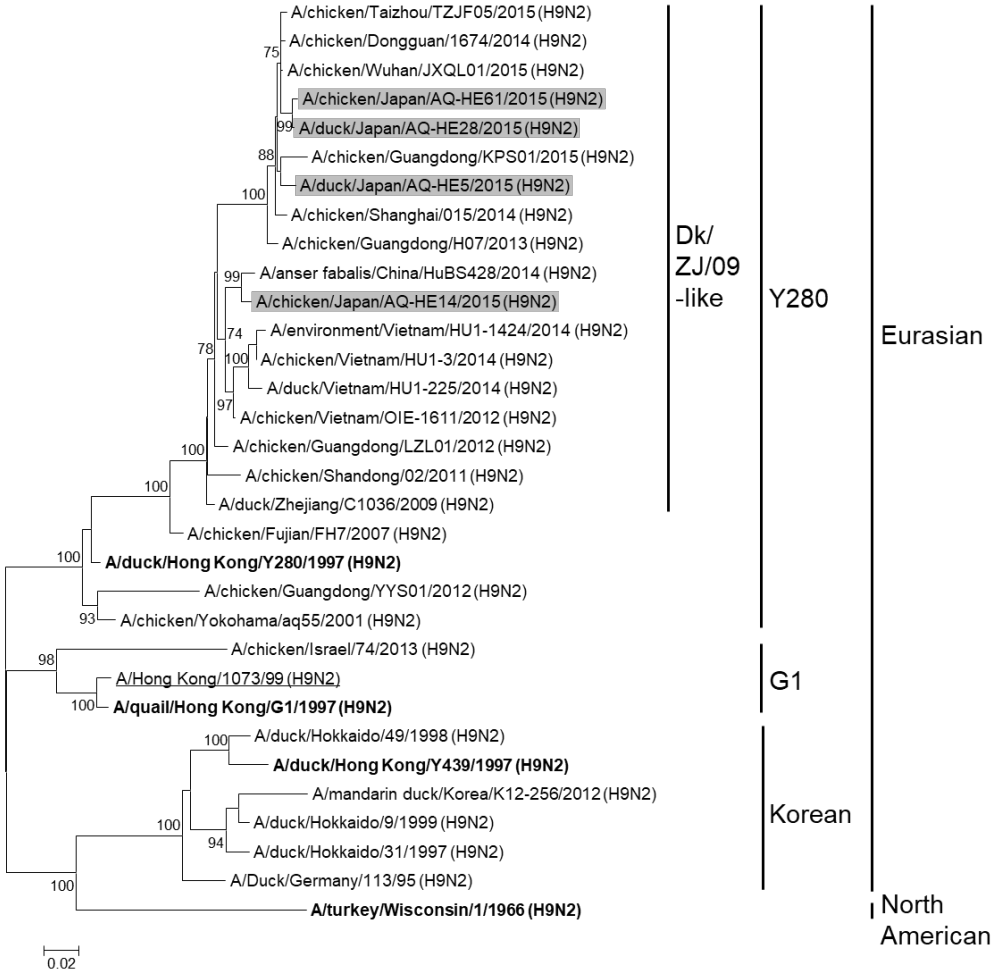



**Figure 3. Phylogenetic tree for the H9 HA genes:** Nucleotides 142-1,077 of the HA gene were analysed using the maximum-likelihood (ML) method with MEGA 7.0 software and divided into two lineages: Eurasian and North American lineages. Eurasian H9 viruses were clustered into three sublineages: Y280, G1, and Y439. Horizontal distances are proportional to the minimum number of nucleotide difference required to join nodes and sequences. Digits at the nodes indicate the probability of confidence levels in a bootstrap analysis with 1,000 replications. Viruses isolated in this study are highlighted in grey, and the representatives of each sublineage are indicated in bold. Isolates from humans are shown underlined.

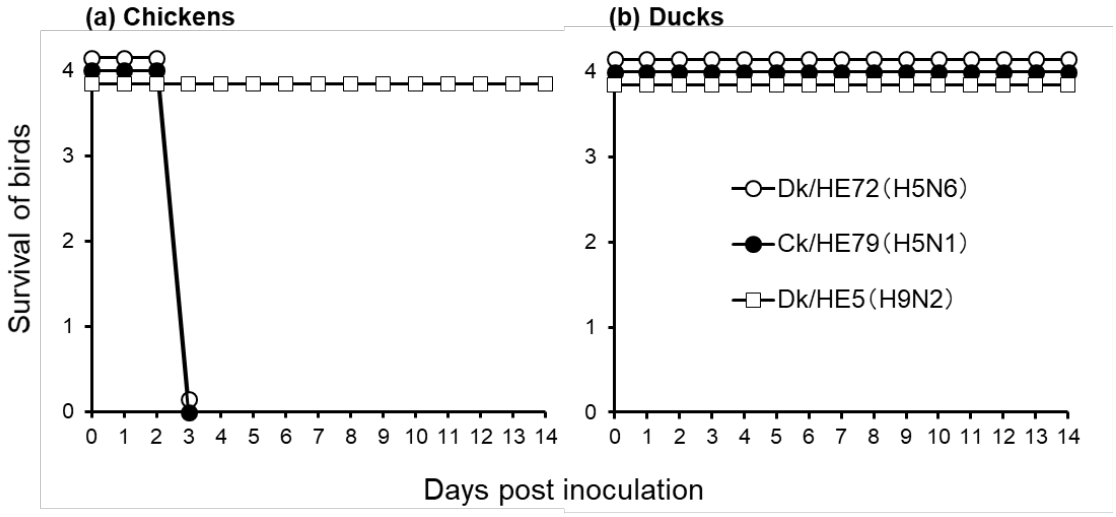



**Figure 4. Survival of chickens (a) and ducks (b) inoculated with each virus:** Four 4-week-old chickens and ducks were inoculated with $10^{6.0}$ $EID_{50}$ of Dk/HE72 (H5N6), Ck/HE79 (H5N1), and Dk/HE144 (H9N2). Survival of inoculated chickens and ducks was observed for 14 days after the challenge.

## Chapter II

## Repeated detection of H7N9 and H7N3 avian influenza viruses in raw poultry meat illegally brought to Japan by international flight passengers

## Introduction

The spread of AIV infections in poultry and humans, such as the H7N9 AIV of novel Asian lineage, identified in eastern China in March 2013, has become a global concern since 2013 (20). Phylogenetic analyses indicate that the H7N9 viruses have been generated as a consequence of multiple reassortment events occurring in domestic and wild birds: their HA and NA genes were derived from H7Nx or HxN9 viruses of Eurasian lineage and six internal genes were from H9N2 viruses of domestic and wild birds in China (63, 64). The H7N9 viruses are classified at least into 36 genotypes (G1–G36), and G1, G3 and G11 are the major genotypes in the 5$^{th}$ wave from October 2016 to September 2017 (65).

Although the H7N9 virus does not easily transmit to humans beyond the host species barrier (mainly because of the difference in the receptor binding site of the HA) (66, 67), patients infected with the virus show severe signs at a high mortality rate, depending on their age (68). Most human cases of infection with H7N9 viruses are due to close contact with infected poultry or contaminated environment such as LBM (69). Except for infection in travelers (70), so far, no report of infection or virus isolation from humans have been made outside mainland China. The emergence of H7N9 HPAIV is not only a concern for animal health but also poses a threat to human health (71).

The migration of wild birds is a major factor in the spread of AIVs over long distances. Although the H7N9 virus has not been isolated from migratory birds in the surveillance and monitoring in surrounding countries (72-74), Zhao *et al*. (75) isolated the virus from an apparently healthy wild tree sparrow that was captured on the Australia-East Asia migratory bird flyway in China.

In addition to migratory birds, many AIVs have been isolated from poultry products transported across the national border (15-19). In the previous chapter, the actual state of contribution of international flight passengers to transportation of infectious AIVs, including H5N1 and H5N6 HPAIVs, was described. During continued inspection at the border, highly and low pathogenic avian influenza viruses of H7N9 subtype and HPAIV of H7N3 subtype were isolated from poultry meat products carried by passengers boarded on flights from China to Japan from 2016 to 2018. This was the first report of the isolation of H7N9 and H7N3 AIVs from raw poultry meat products outside mainland China. In this Chapter, these H7N9 and H7N3 virus isolates were characterized through genetic and antigenic analyses, and animal experiments were conducted in order to understand the pathogenicity and tissue tropism of the virus in chickens and ducks.

## Materials and Methods

***Poultry meat products used for virus isolation***

AQS collected 163 specimens of poultry meat products (some products include organs) that were confiscated at Tokyo Narita Airport, Tokyo Haneda Airport, Kansai Airport, Chubu Airport, New Chitose Airport, Sendai Airport, Hiroshima Airport, Naha Airport and Osaka Port from May 2016 to March 2018 (Table 7). Species of poultry samples were identified by visual inspection and sequencing of the mitochondrial DNA, as described previously (31).

***Virus isolation***

All 163 confiscated samples shown in Table 7 were used for virus isolation, which was performed as described in Chapter I.

***Viruses used in antigenic analysis***

Viruses were grown in 10-day-old embryonated chicken eggs, and infectious allantoic fluids were stored at −80°C until use. A/Anhui/1/2013 (H7N9) was kindly provided by Dr. Masato Tashiro, National Institute of Infectious Diseases, Japan. A/chicken/North Korea/7916/2005 (H7N7) and A/chicken/New South Wales/327/1997 (H7N2) were kindly provided by Dr. Paul Selleck, Australian Animal Health Laboratory. A/turkey/Italy/4580/1999 (H7N1) was kindly provided by Dr. Ilaria Capua, Istituto Zooprofilattico Sperimentale delle Venezie. A/Seal/Massachusetts/1/1980 (H7N7) was kindly provided by Dr. Webster, St. Jude Children's Research Hospital, Tennessee, USA (76). A/mallard/Netherlands/12/2000 (H7N3) was kindly provided by Dr. AD. Osterhaus, Erasmus University. A/Guangdong/17SF003/2016 (H7N9) was kindly provided by Drs. Y. Shu and D. Wang, Chinese Center for Disease control and Prevention. A/duck/Hokkaido/Vac-2/2004 (H7N7) was generated from two nonpathogenic avian influenza viruses isolated from migratory ducks and was obtained as a vaccine candidate strain against H7N7 virus (77). A/duck/Hokkaido/1/2010 (H7N7), A/duck/Hokkaido/W19/2013 (H7N2) and A/duck/Taiwan/Ya103/1993 (H7N7) were representative strains in H7 Far-Eastern sublineage, European-Asian sublineage and H7 Historical Europe lineage, respectively (41).

***Genetic analysis***

All the AIV isolates in this chapter were sequenced and subjected to phylogenetic analysis. Viral RNA was extracted from the infectious allantoic fluid using QIAamp viral RNA mini kit. MiSeq libraries were prepared using Next Ultra RNA

Library Prep Kit and Multiplex Oligos for Illumina and sequenced on the MiSeq, using MiSeq reagent kit v3. Reads were *de novo* assembled using CLC Genomic Workbench.

Nucleotide sequences were phylogenetically analyzed by the maximum-likelihood method with Tamura–Nei model and bootstrap analysis ($n$ = 1,000) using MEGA 7.0 software (40) with default parameters. The sequences of each segments of the H7 and H9 virus isolates obtained in the present study were compared with those of other strains deposited in public databases, GenBank/EMBL/DDBJ and GISAID, and incorporated into the phylogenetic analysis. The gene sequences in the present study have been registered in GenBank/EMBL/DDBJ as indicated in Table 8.

***Antigenic analysis***

Antigenic properties of H7N9 virus isolates, A/duck/Japan/AQ-HE28-3/2016 (Dk/HE28-3) (H7N9) and A/duck/Japan/AQ-HE29-22/2017 (Dk/HE29-22) (H7N9), were determined via the HI test using polyclonal antisera, prepared from chickens (*G. gallus*, Julia) immunized with reference H7 AIV strains (41, 78, 79). Antigenic properties of A/duck/Japan/AQ-HE30-1/2018 (Dk/HE30-1) (H7N3) was determined via the HI test using polyclonal antisera, prepared from ferret immunized with reference H7 AIV strains. The HI test was performed as previously reported (79). Test antisera were mixed with 10% chicken red blood cell suspension for 16 h at 4°C, and the centrifuged supernatant was diluted with PBS. The diluted test antisera of 0.025 ml were mixed with equal volume of the test virus with a concentration of 8 HA units per 0.05 ml and incubated for 30 min at room temperature. The 0.5% chicken red blood cell suspension of 0.05 ml was added, and they were incubated for 60 min at room temperature. HI titre was decided as the reciprocal of the highest dilution of antisera that completely inhibited hemagglutination.

***IVPI test for H7N9 and H7N3 virus isolates***

The pathogenic phenotype of Dk/HE29-22 (H7N9) and Dk/HE30-1 (H7N3) were assessed in 10 six-week-old chickens (*G. gallus*, Julia), which were obtained from Hokkai Starchick, Hokkaido, Japan. Virus stock with 64 HA titre had been diluted 1:10 in sterile PBS before injection. Each of 10 chickens was inoculated intravenously with 0.1 ml of diluted virus stock, and clinical symptoms were observed and scored for 10 days. Clinical score was given to each chicken based on observation: score 0 if normal, 1 if sick, 2 if severely sick and 3 if dead. Intravenous pathogenicity index (IVPI) was decided as the mean score per bird per 10 days observation (8).

***Intranasal inoculation experiment for H7N9 virus isolates***

Dk/HE29-22 (H7N9) and Dk/HE28-3 (H7N9) were selected for animal experiments. Four-week-old chickens (*G. gallus*, Julia) were obtained from Hokkai Starchick, Hokkaido, Japan. Four-week-old domestic ducks (*Anas platyrhynchos var. domesticus,* Cherry Valley) were obtained from Takikawa Shinseien, Hokkaido, Japan. Eight birds were used in each experiment and randomly divided into two groups of four birds, sample-collection and observation groups, to assess the pathogenicity and tissue tropism in birds. All chickens and ducks were intranasally inoculated with 0.1 ml of virus solution containing $10^{6.0}$ $EID_{50}$ of Dk/HE29-22 (H7N9) or Dk/HE28-3 (H7N9). The birds of the sample-collection group were euthanized at 3 dpi for the collection of trachea swab, cloacal swab, blood, brain, trachea, lung, kidney, colon and breast meat samples. To prepare the 10% suspension with MEM, tissue samples were homogenized using a Multi-Beads Shocker. The infectivity for each swab, blood, and tissue sample was titrated via $EID_{50}$ using embryonated chicken eggs. The birds of the observation group were observed clinically until 14 dpi. All infected birds were kept in self-contained isolator units at the BSL3 biosafety facility at the Faculty of Veterinary Medicine, Hokkaido University, Japan.

***Ethics statements***

All animal experiments were authorized by the Institutional Animal Care and Use Committee of the Graduate School of Veterinary Medicine, Hokkaido University (approval number: 13-0108). All experiments were performed according to the guidelines of the committee.

## Results

***Isolation of AIVs from poultry products illegally carried into the airports in Japan***

Seven AIVs (three H7N9 and one H7N3 and three H9N2 subtypes) were isolated from meat- and organ-pooled samples of chicken (*Gallus gallus*) or Muscovy duck (*Cairina moschata*) meat products brought by passengers who were boarded on flights from China and Vietnam to Japan in 2016–2018, as listed in Table 8. All embryonated chicken eggs inoculated with the meat- and organ-pooled samples, from which Dk/HE29-22 (H7N9) or Dk/HE29-52 (H7N9) was isolated, died within 48 hours. The infectivity for each meat- and organ-pooled sample was titrated via $EID_{50}$ using embryonated chicken eggs. Virus titres of these pooled samples ranged from $\leq 10^{1.5}$ to $10^{3.8}$ $EID_{50}$/g (Table 8). In the titres of $\leq 10^{1.5}$, viruses were isolated, but the titre was less than the calculation limit of $EID_{50}$.

***Genetic analysis of H7N9 virus isolates***

The complete genome sequences of three H7N9 virus isolates, Dk/HE28-3 (H7N9), Dk/HE29-22 (H7N9) and Dk/HE52-22 (H7N9) (available at the GenBank/EMBL/DDBJ), were determined to analyze genetic relationship with other H7N9 AIVs. Phylogenetic analysis of the HA gene revealed that Dk/HE28-3 (H7N9) was classified into the genetic clade W4/5-3 of the Yangtze River Delta Lineage, and Dk/HE29-22 (H7N9) and Dk/HE29-52 (H7N9) were classified into the genetic clade W4/5-4 of the Yangtze River Delta Lineage, based on the recent classification system (Figure 5) (80-82). All eight gene segments of Dk/HE28-3 (H7N9) shared 97.2%–99.8% nucleotide sequence identity with those of A/duck/Zhejiang/S1375/2016 (H7N9), isolated in eastern China. All eight gene segments of Dk/HE29-22 (H7N9) and three gene segments (HA, NA and MP genes) of Dk/HE29-52 (H7N9) shared 99.4%–100.0% nucleotide sequence identity with A/chicken/Heinan/ZZ01/2017 (H7N9), isolated in central China, and the other five internal gene segments of Dk/HE29-52 (H7N9) shared >97.3% nucleotide sequence identity with AIVs isolated from waterfowls in east Asia or Alaska (Figure 6). Dk/HE29-52 (H7N9) is a reassortant virus that contains the PB2, PB1, PA, NP and NS genes derived from wild waterfowls. Dk/HE29-22 (H7N9) and Dk/HE29-52 (H7N9) were confirmed to possess multiple basic amino acid residues, PEVPKRKRTAR/GLF, at the cleavage site in the HA, which is the major motif of H7N9 HPAIVs (65, 71). In the putative amino acid sequences, Dk/HE29-22 (H7N9) and Dk/HE29-52 (H7N9) possess the amino acid sequence QSG at positions 226–228 (H3 numbering) of the HA, which is associated with specificity to the avian-type receptor (83). In contrast, Dk/HE28-3 (H7N9) contains the amino acid 226L in the HA as

observed in many H7N9 LPAIVs, which is known to increase binding to the human-type receptor. The amino acid 368V in PB1, which is closely associated with the virulence and transmissibility of the virus in ferrets, was observed in Dk/HE29-22 (H7N9) and Dk/HE28-3 (H7N9), as it has been in H7N9 AIVs since 2013, but Dk/HE29-52 did not have this amino acid (82). According to the recent genotype classification by Quan et al. (65), Dk/HE28-3 (H7N9) and Dk/HE29-22 (H7N9) were classified into G11 (A/Anhui/40094/2015-like) and G3 (A/chicken/Zhongshan/ZS/2017-like) genotype, respectively. However, Dk/HE29-52 (H7N9) is not classified into either of G1–G36 genotypes.

***Genetic analysis of H7N3 virus isolate***

The complete genome sequence of Dk/HE30-1 (H7N3) was determined to understand its genetic properties and to analyze its genetic relationship with other AIVs that have been isolated in North and South America, Europe, and Asia and listed in GenBank/EMBL/DDBJ and GISAID. Sequence analysis showed that the HA and MP gene segments of Dk/HE30-1 (H7N3) shared 98.6–99.5% nucleotide sequence identity with those of A/chicken/Heinan/ZZ01/2017 (H7N9) isolated in central China, while its NA gene segment shared 99.1% nucleotide sequence identity with that of A/environment/Fujian/S1XA33/2017 (H11N3) isolated in poultry slaughterhouses in southeast China. Its remaining five internal gene segments were closely related to those of other AIVs isolated from poultry originating from China, i.e., the PB2 gene segment of Dk/HE30-1 (H7N3) shared 97.8% nucleotide sequence identity with that of A/duck/Ganzhou/GZ148/2016 (H6N6); the PB1 and NP gene segments of Dk/HE30-1 (H7N3) shared 97.6–98.8% nucleotide sequence identity with those of A/duck/Japan/AQ-HE103/2015 (H1N2), which was isolated from a duck meat product that was illegally brought to Japan by a flight passenger from China as described in Chapter I; the PA gene segment of Dk/HE30-1 (H7N3) shared 97.6% nucleotide sequence identity with that of A/duck/Guangxi/135D20/2013 (H3N2); and the NS gene segment of Dk/HE30-1 (H7N3) shared 98.2% nucleotide sequence identity with that of A/chicken/Guangxi/125C8/2012 (H3N2). Phylogenetic analysis classified Dk/HE30-1 (H7N3) into the same clade as the H7N9 HPAIVs in the Yangtze River Delta Lineage based on the HA gene (Figure 7A) and into the same clade as HxN3 AIVs that have recently been isolated from poultry or the environment in China based on the NA gene (Figure 7B), demonstrating that Dk/HE30-1 (H7N3) was a multiple reassortant virus that contained the HA gene of H7N9 HPAIVs.

***Genetic analysis of H9 virus isolates***

To clarify the genetic relationship of H9N2 AIVs, the complete genome sequences of three H9N2 virus isolates were determined: A/chicken/Japan/AQ-HE28-28/2016 (Ck/HE28-28) (H9N2), A/chicken/Japan/AQ-HE28-50/2016 (Ck/HE28-50) (H9N2), and A/chicken/Japan/AQ-HE28-57/2016 (Ck/HE28-57) (H9N2) (available at the GenBank/EMBL/DDBJ). Phylogenetic analysis of the HA gene showed that all three isolates belonged to the Y280 sublineage of the Eurasian lineage and were closely related to H9N2 viruses isolated from poultry in China and Vietnam (Figure 8). Ck/HE28-28 (H9N2) was closely related to A/chicken/Yuhuan/YH15/2016 (H9N2), isolated in eastern China, with identities ranging from 96.9% to 99.7% for all eight gene segments. Ck/HE28-50 (H9N2) was closely related to H9N2 viruses isolated from poultry in Vietnam, with identities >98.3% in each segment, except for the NS gene, which shared >98.5% nucleotide identity with H9N2 viruses isolated from poultry in eastern China. Ck/HE28-57 (H9N2) was closely related to H9N2 viruses isolated from poultry in Vietnam, with similarities >97.4% in each segment. The cleavage site of HA protein of all H9N2 isolates contained a PSRSSR/GLF motif (Table 8), corresponding to the cleavage site motif of H9N2 viruses isolated in China in recent years (49).

***Antigenic analysis of representative H7N9 virus isolates***

Dk/HE29-22 (H7N9) and Dk/HE28-3 (H7N9) were antigenically analyzed by HI test, using a panel of polyclonal chicken antisera (Table 9). Chicken antisera against A/Anhui/1/2013 (H7N9), A/duck/Hokkaido/W19/2013 (H7N2) and old H7 virus strains isolated in the 1990s (A/duck/Taiwan/Ya103/1993 (H7N7), A/chicken/New South Wales/327/1997 (H7N2) and A/turkey/Italy/4580/1999 (H7N1)) effectively inhibited hemagglutination of Dk/HE28-3 (H7N9). On the contrary, the antisera against the above strains showed low inhibition of hemagglutination of Dk/HE29-22 (H7N9), in comparison with that of Dk/HE28-3 (H7N9), indicating that the HA antigenicity of Dk/HE29-22 (H7N9) slightly drifted from that of A/Anhui/1/2013 (H7N9) or Dk/HE28-3 (H7N9).

***Antigenic analysis of representative H7N3 virus isolate***

Dk/HE30-1 (H7N3) was antigenically analyzed by cross HI test, using a panel of post-infection ferret antisera (Table 10). The ferret antiserum against A/Anhui/1/2013 (H7N9) reacted equally with Dk/HE28-3 (H7N9), a low pathogenic H7N9 strain of Yangtze River Delta (YRD) linage to the homologous antigen, and that against A/Guangdong/17SF003/2016 (H7N9) did so with Dk/HE29-22 (H7N9) and Dk/HE29-52 (H7N9), highly pathogenic H7N9 strains of YRD HPAI linage. On the contrary,

those against A/Anhui/1/2013 (H7N9) and A/Guangdong/17SF003/2016 (H7N9) showed more than 16-fold and 4-fold lower HI titres, respectively, with Dk/HE30-1 (H7N3), indicating that the HA antigenicity of Dk/HE30-1 (H7N3) slightly differed from that of A/Anhui/1/2013 (H7N9) and A/Guangdong/17SF003/2016 (H7N9).

***Pathogenicity of representative H7N9 and H7N3 virus isolates in chickens***

To assess the pathogenicity of Dk/HE29-22 (H7N9) and Dk/HE30-1 (H7N3), their IVPI were determined, according to the manual of the World Organization for Animal Health (8). Ten 6-week-old chickens were intravenously inoculated with the allantoic fluid containing Dk/HE29-22 (H7N9) or Dk/HE30-1 (H7N3) and monitored for 10 days. All chickens died within 3 dpi, and the values of IVPI for Dk/HE29-22 (H7N9) and Dk/HE30-1 (H7N3) were 2.88 and 2.99 respectively, based on the score of clinical signs.

To examine their pathogenicity and tissue tropism in chickens, each of Dk/HE28-3 (H7N9) and Dk/HE29-22 (H7N9) was intranasally administered in eight chickens which were divided into two groups of four chickens for sample-collection and observation groups. In the chickens of the sample-collection group inoculated with Dk/HE28-3 (H7N9), virus was recovered from each of the four chickens. The frequencies of virus detection in the samples were high for tracheas (4/4), trachea swabs (4/4), lungs (3/4) and colons (3/4), while they were low for kidney (1/4) and muscle (1/4). No virus was recovered from blood, brain tissue or cloacal swab samples (Table 11). The geometric mean (GM) virus titres of positive samples, were higher in the samples of the respiratory organs, $10^{5.2}$ $EID_{50}$/ml in trachea swabs, $10^{4.6}$ $EID_{50}$/g in trachea and $10^{4.5}$ $EID_{50}$/g in lungs, compared with that in kidney, colon and muscle samples (ranging from $\leq 10^{2.0}$ to $10^{3.0}$ $EID_{50}$/g). In the chickens of the sample-collection group inoculated with Dk/HE29-22 (H7N9), all chickens died on 3 dpi, and swabs and tissue samples were taken from dead chickens. Virus was recovered from each of the samples of four dead chickens, and the GM virus titres of each sample were $10^{7.0}$ $EID_{50}$/g in brain, $10^{7.0}$ $EID_{50}$/g in trachea, $10^{7.3}$ $EID_{50}$/g in lungs, $10^{7.7}$ $EID_{50}$/g in kidney, $10^{6.4}$ $EID_{50}$/g in colon, $10^{5.6}$ $EID_{50}$/g in trachea swab and $10^{4.1}$ $EID_{50}$/g in cloacal swab (Table 11). In the chickens of the observation group, all chickens inoculated with Dk/HE28-3 (H7N9) survived for 14 days without exhibiting any clinical signs, while each of the chickens inoculated with Dk/HE29-22 (H7N9) showed depression and anorexia and died on 3 dpi (Figure 9a).

***Pathogenicity of representative H7N9 virus isolates in ducks***

To assess the pathogenicity and tissue tropism in ducks, each of Dk/HE28-3 (H7N9) and Dk/HE29-22 (H7N9) was intranasally administered in eight ducks which were divided into two groups of four ducks for sample-collection and observation groups. In the ducks of the sample-collection group inoculated with Dk/HE28-3 (H7N9), virus was recovered only from the respiratory tract of two ducks. The frequencies of virus recovery in the samples were 2/4 of the lungs, 2/4 of the tracheal swabs, and 1/4 of the trachea, and each virus titre in positive samples was close to the detection limit (Table 11). In the ducks of the sample-collection group inoculated with Dk/HE29-22 (H7N9), virus was recovered from all of the samples from one duck and some tissue samples of the remaining three ducks. One of the ducks had systemic infection demonstrated high viral titres ($10^{7.7}$ $EID_{50}$/g in lungs) without showing any clinical signs. Two ducks of this group demonstrated low virus titres in the local tissue, trachea, muscle and colon, and the other duck demonstrated higher virus titres in the kidney and muscle samples than in the lung tissue sample. Some individual differences were observed in virus recovery from tissues of ducks compared with chickens (Table 11). In the ducks of the observation group, all ducks inoculated with Dk/HE29-22 (H7N9) or Dk/HE28-3 (H7N9) survived for 14 days without showing any clinical signs (Figure 9b).

## Discussion

In Chapter I, it was revealed that H5 HPAIV-contaminated meat products were brought into the international airports by flight passengers in their hand luggage, which suggested that they must play a role in transporting infectious HPAIVs abroad. As in the case of wild birds, continued monitoring of AIVs in meat products brought across the border is effective in raising passengers' awareness and predicting the risk of AIV infections.

Three H7N9, one H7N3 and three H9N2 AIVs were isolated from poultry products illegally brought to Japan by international flight passengers as the result of this continuous monitoring from May 2016 to March 2018. Phylogenetic analysis showed that all of the virus isolates were closely related to AIVs isolated in eastern China or Vietnam in recent years. The two Muscovy duck meat products, from which Dk/HE29-22 (H7N9) or Dk/HE29-52 (H7N9) were isolated, were transported in the hand luggage of flight passengers who had boarded international flights at Fuzhou, China, and were detected at Tokyo Narita Airport, Japan, by quarantine detector dogs. It was found from interviewing the carriers of these contaminated products that each of the two Muscovy duck meat products originated from backyard breeding poultry flocks. The slaughtering of poultry in backyard or wholesale market environments (where veterinary supervision and hygiene management are generally insufficient compared with public slaughterhouses) might be one of the leading causes of virus circulation in the region (84). Unlike chickens, ducks inoculated with HPAIV Dk/HE29-22 (H7N9) discharged viruses in major organs and muscle tissues without showing any symptoms, suggesting that the Muscovy ducks infected with Dk/HE29-22 (H7N9) were slaughtered for consumption without symptoms and carried long distance while contaminated with virus. As well as by the transportation of live poultry, H7N9 AIVs may be spread via infected poultry products. Since the numbers of detected cases of illegal importation of meat products by passengers are increasing in Japan, it is important for border control to maintain continued vigilance.

The results from the intranasal inoculation experiment, HPAIV Dk/HE29-22 (H7N9) is thought to have been adapted in chickens compared with LPAIV Dk/HE28-3 (H7N9). Viral adaptation to chickens should be gained through the accumulation of genetic mutation by repeated passage in chickens, which could allow AIVs to acquire high pathogenicity (85-87) or virus replication efficiency in chickens (88). Compared with the previous study which was performed using the same protocol, equipment and facilities as this study, the frequency of virus recovery and GM virus titre in the tissues of chickens inoculated with Dk/HE28-3 (H7N9) were higher than those of chickens

inoculated with A/Anhui/1/2013 (H7N9), the representative strain isolated in 2013 (53). Moreover, the frequency of virus recovery and GM virus titre in the samples of chickens inoculated with HPAIV Dk/HE29-22 (H7N9) was even higher than those of chickens inoculated with Dk/HE28-3 (H7N9). The virus replication efficiency of Dk/HE29-22 (H7N9) or Dk/HE28-3 (H7N9) in chickens is assumed to be increased during circulation in chicken flocks, which indicates that the adaptation of novel H7N9 viruses to chickens has progressed steadily in the field chicken flocks and has led to the emergence of the novel H7N9 HPAIVs by the presumed mechanism, as previously reported (86). In addition to chickens, the virus replication efficiency of HPAIV Dk/HE29-22 (H7N9) in ducks was also higher than that of LPAIV Dk/HE28-3 (H7N9), indicating the increasing risk of spreading virus intercontinentally via migratory birds (89, 90).

In contrast to Dk/HE28-3 (H7N9), antigenicity of the HA of Dk/HE29-22 (H7N9) was slightly different from that of A/Anhui/1/2013 (H7N9), A/duck/Hokkaido/W19/2013 (H7N7), and older H7 HPAIV strains. In addition, the antigenicity of the HA of Dk/HE30-1 (H7N3) was also slightly different from that of A/Anhui/1/2013 (H7N9) and A/Guangdong/17SF003/2016 (H7N9), indicating that the antigenic drift of the novel H7Nx HPAIV might have progressed in the field. The antigenic variation of AIV is mainly attributed to amino acid change in the globular head region of the HA protein, and vaccination in poultry is thought to be the main driver of antigenic drift of AIVs because of its contribution to generate immune-escaping viral mutants (91). The progress of antigenic variation might lead to further human infection and outbreaks in poultry, such as in the case of the A/goose/Guangdong/1/1996 lineage H5 HPAIVs that have been demonstrated antigenic diversity (44, 92) and spread in human and bird populations around the world. Further precise antigenic analysis of H7Nx viruses is necessary to monitor the antigenic diversity of the viruses.

AIVs isolated from poultry are thought to have evolved gradually from wild-bird-derived AIVs during the successive adaptation process in poultry, especially quails or domestic ducks (88, 93). AIVs isolated from poultry products can replicate efficiently and have a high possibility of being shed into the environment when they are carried into poultry farms. In addition to the risk of infection to the poultry, repeatedly bringing contaminated meat products into a gathering place increases the risk of human infection. Although the HA amino acid 226L have mutated back to 226Q in the novel H7Nx HPAIVs, some reports have suggested that the H7N9 HPAIVs have retained human-type and avian-type receptor binding properties (94) and possessed the ability to

transmit among ferrets (95) as observed in the H7N9 LPAIVs. Further study is necessary to analyze these mammalian adaptation of the novel H7Nx viruses.

Illegal importation of meat products in hand luggage by international passengers would continue to occur at any port and airport, regardless of the implementation of a variety of border control measures. To prevent passengers carrying infectious AIVs via contaminated products, it is important to increase publicity and awareness to ensure that passengers, particularly from areas where the virus is prevalent, comply with import regulations. Continued control and monitoring at the border are essential for the prevention of virus circulation and spread, as is disease control based on the detection and culling of infected poultry in endemic areas.

## Summary

H7N9 highly and low pathogenic avian influenza viruses and H7N3 HPAIV have been isolated from duck meat products that were brought illegally into Japan by flight passengers in their hand luggage. These novel H7Nx virus isolates were phylogenetically closely related to those prevailing in China. Antigenic analysis revealed that the hemagglutinin of the H7N9 and H7N3 HPAIV isolates were slightly different from those of the H7N9 LPAIV and older H7 strains. Experimental infections of H7N9 HPAIV or LPAIV isolates in chickens or ducks demonstrated that the virus replication efficiency of H7N9 HPAIV isolate was higher than that of H7N9 LPAIV isolate, indicating the risk of the virus spreading is increasing. These meat products contaminated with AIVs repeatedly brought into Japan lead to increased risks of poultry and public health. Continuous border disease control based on the detection and culling of infected poultry and meat products is, thus, essential for the prevention of introduction and spread of AIVs.

**Table 7.** Total number of illegally carried poultry products investigated from May 2016 to March 2018

| Country or region of loading[a] | Meat products | | | Total |
|---|---|---|---|---|
| | Chicken | Duck | Quail | |
| China | 33 | 61 | - | 94 |
| Vietnam | 46 | 6 | 2 | 54 |
| Korea | 6 | 2 | - | 8 |
| Taiwan | 5 | - | - | 5 |
| Russia | - | 1 | - | 1 |
| United Arab Emirates | 1 | - | - | 1 |
| Total | 91 | 70 | 2 | 163 |

[a]The last country or region of loading in case of transship

-: None

**Table 8.** Avian influenza viruses isolated from poultry meat products

| Land of loading[a] | Arrival day[b] | Species of poultry | Names | Titre of isolated meat product (log $EID_{50}$/g) | Cleavage site of haemagglutinin | Accession numbers |
|---|---|---|---|---|---|---|
| Fuzhou (China) | 05/23/2016 | *Cairina moschata* | A/duck/Japan/AQ-HE28-3/2016 (H7N9) | 2.5 | PEIPKGR-GLF | LC374914-21 |
| Nanjing (China) | 06/16/2016 | *Gallus gallus* | A/chicken/Japan/AQ-HE28-28/2016 (H9N2) | ≤2.0[c] | PSRSSR -GLF | LC374922-29 |
| Ho Chi Minh (Vietnam) | 08/09/2016 | *Gallus gallus* | A/chicken/Japan/AQ-HE28-50/2016 (H9N2) | 2.5 | PSRSSR -GLF | LC374930-37 |
| Hanoi (Vietnam) | 08/09/2016 | *Gallus gallus* | A/chicken/Japan/AQ-HE28-57/2016 (H9N2) | ≤1.5[b] | PSRSSR-GLF | LC374938-45 |
| Fuzhou (China) | 06/23/2017 | *Cairina moschata* | A/duck/Japan/AQ-HE29-22/2017 (H7N9) | 3.0 | PEVPKRKRTAR-GLF | LC315921-28 |
| Fuzhou (China) | 09/21/2017 | *Cairina moschata* | A/duck/Japan/AQ-HE29-52/2017 (H7N9) | 3.8 | PEVPKRKRTAR-GLF | LC374946-53 |
| Lanzhou/Shanghai (China) | 03/01/2018 | *Cairina moschata* | A/duck/Japan/AQ-HE30-1/2018 (H7N3) | NT | PEVPKRRRTAR-GLF | LC416563-70 |

[a]The last land in case of transhipment

[b]Arrival date of poultry meat products at airport in Japan.

[c]Viruses were isolated, but the titre is not greater than the calculation limit of $EID_{50}$.

NT: Not tested

**Table 9.** Antigenic analysis of H7 influenza viruses with antisera using hemagglutination inhibition test

| Viruses | Subtypes | Antiserum to | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Dk/Hok/ Vac-2/04 | Anhui/ 1/13 | Ck/NK/ 7916/05 | Dk/Hok/ W19/13 | Ty/Italy/ 4580/99 | Dk/Tw/ Ya103/93 | Ck/NSW/ 327/97 | Sl/Mass/ 1/80 |
| **A/duck/Japan/AQ-HE29-22/2017** [a] | H7N9 | 320 | 640 | 640 | 640 | 320 | 80 | 640 | 40 |
| A/duck/Japan/AQ-HE28-3/2016 [a] | H7N9 | 5,120 | 2,560 | 5,120 | 5,120 | 1,280 | 1,280 | 5,120 | 160 |
| A/duck/Hokkaido/1/2010 | H7N7 | 5,120 | 1,280 | 2,560 | 10,240 | 1,280 | 640 | 5,120 | 320 |
| A/duck/Hokkaido/Vac-2/2004 | H7N7 | 20,480 | 2,560 | 5,120 | 20,480 | 2,560 | 1,280 | 10,240 | 1,280 |
| A/Anhui/1/2013 | H7N9 | 10,240 | 5,120 | 5,120 | 5,120 | 2,560 | 640 | 5,120 | 320 |
| **A/chicken/North Korea/7916/2005** | H7N7 | 640 | 1,280 | 1,280 | 5,120 | 1,280 | 320 | 2,560 | 160 |
| A/duck/Hokkaido/W19/2013 | H7N2 | 5,120 | 1,280 | 1,280 | 2,560 | 1,280 | 320 | 2,560 | 320 |
| **A/turkey/Italy/4580/1999** | H7N1 | 160 | 80 | 320 | 320 | 1,280 | 80 | 320 | 80 |
| **A/duck/Taiwan/Ya103/1993** | H7N7 | 160 | 160 | 320 | 320 | 80 | 2,560 | 160 | 40 |
| **A/chicken/New South Wales/327/1997** | H7N2 | 1,280 | 640 | 1,280 | 5,120 | 1,280 | 320 | 5,120 | 320 |
| A/seal/Massachusetts/1/1980 | H7N7 | 20,480 | 2,560 | 10,240 | 10,240 | 2,560 | 320 | 10,240 | 2,560 |

[a]Viruses isolated in this study

Homologous titres are underlined.

HPAIVs are shown in bold.

**Table 10.** Hemaggulutination inhibition assay of A(H7) influenza viruses.

| Viruses | Subtype | Lineage | Antiserum to | | |
|---|---|---|---|---|---|
| | | | Ma/NL12 | Anhui1 | GD17SF003 |
| A/mallard/Netherlands/12/2000 | H7N3 | - | <u>320</u> | 40 | 320 |
| A/Anhui/1/2013 | H7N9 | - | 160 | <u>160</u> | 160 |
| **A/Guangdong/17SF003/2016** | H7N9 | YRD HPAI | 20 | 10 | <u>160</u> |
| A/duck/Japan/AQ-HE28-3/2016 | H7N9 | YRD | 80 | 160 | 640 |
| **A/duck/Japan/AQ-HE29-22/2017** | H7N9 | YRD HPAI | 20 | 20 | 160 |
| **A/duck/Japan/AQ-HE29-52/2017** | H7N9 | YRD HPAI | 20 | 20 | 160 |
| **A/duck/Japan/AQ-HE30-1/2018** | H7N3 | YRD HPAI | 10 | <10 | 40 |

Homologous titres are underlined.

HPAIVs are shown in bold.

**Table 11.** Virus recovery at 3 days post-inoculation from chickens and domestic ducks inoculated with each virus.

| Species | Viruses | Sampling day | Swabs (log $EID_{50}$/ml) | | Blood (log $EID_{50}$/ml) | Tissue samples (log $EID_{50}$/g) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | trachea | Cloacal | | Brain | Trachea | Lungs | Kidneys | Colon | Muscle |
| Chickens | Dk/HE28-3 (H7N9) | 3 | 6.3 | - | - | - | 6.8 | 6.8 | - | - | ≤2.0 |
| | | 3 | 5.0 | - | - | - | 4.3 | - | - | ≤2.0 | - |
| | | 3 | 4.5 | - | - | - | 3.0 | 2.8 | - | ≤2.0 | - |
| | | 3 | 5.0 | - | - | - | 5.0 | 4.8 | 3.0 | 5.3 | - |
| | Dk/HE29-22 (H7N9) | 3† | 4.5 | 3.5 | NT | 6.8 | 7.5 | 7.8 | 6.3 | 4.5 | NT |
| | | 3† | 5.8 | 4.5 | NT | 6.5 | 5.5 | 6.5 | 9.8 | 8.5 | NT |
| | | 3† | 6.3 | 3.8 | NT | 7.3 | 7.8 | 7.3 | 7.5 | 6.5 | NT |
| | | 3† | 5.8 | 4.8 | NT | 7.3 | 7.5 | 7.5 | 7.5 | 6.8 | NT |
| Ducks | Dk/HE28-3 (H7N9) | 3 | ≤0.8 | - | - | - | ≤2.0 | ≤2.0 | - | - | - |
| | | 3 | - | - | - | - | - | - | - | - | - |
| | | 3 | - | - | - | - | - | - | - | - | - |
| | | 3 | ≤0.8 | - | - | - | - | ≤2.0 | - | - | - |
| | Dk/HE29-22 (H7N9) | 3 | - | - | - | - | ≤1.8 | - | - | - | ≤1.8 |
| | | 3 | - | - | - | - | - | - | - | ≤1.8 | - |
| | | 3 | 3.0 | ≤1.0 | 2.7 | ≤2.0 | 3.8 | 7.7 | 5.0 | 4.5 | 2.7 |
| | | 3 | - | - | - | - | - | ≤2.3 | 5.0 | - | 3.5 |

-: <0.8 (swabs and blood); <1.8 (tissue samples)

†: Day of death

NT: Not tested

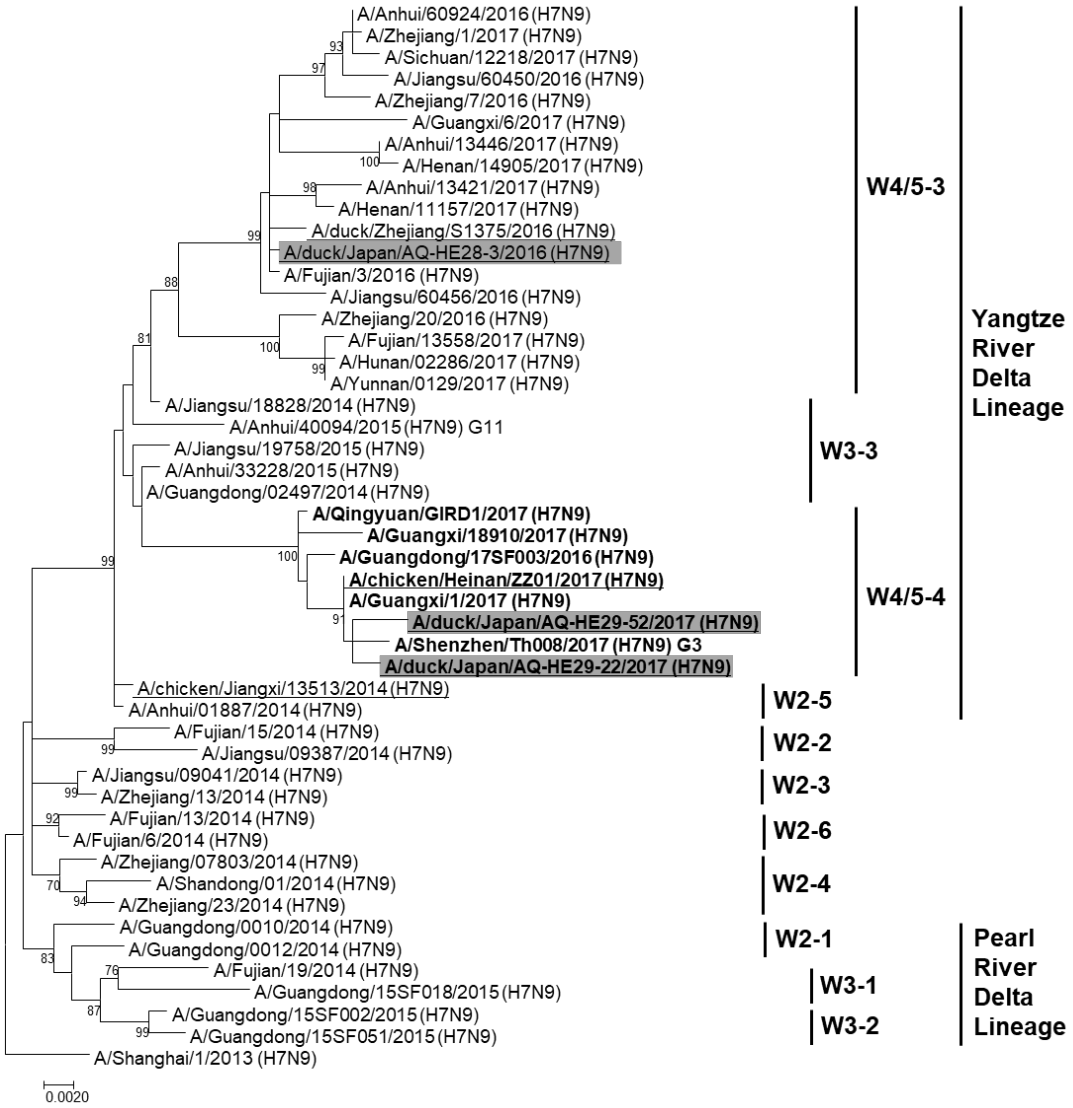



**Figure 5. Phylogenetic trees of the HA gene segments of H7N9 highly pathogenic and low pathogenic avian influenza viruses isolated in Japan:** The nucleotide sequences of the H7 HA genes were analyzed by the maximum-likelihood method along with the corresponding genes of reference strains using MEGA 7.0 software (http://www.megasoftware.net/). The horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. Digits at the nodes indicate confidence levels in a bootstrap analysis with 1,000 replications. The viruses isolated in this study are highlighted in gray. The viruses isolated in birds are underlined. Highly pathogenic avian influenza viruses are indicated in bold. The H7 HA gene sequences are classified into genetic clades as defined by Su *et al*. (2017) or WHO (2017).

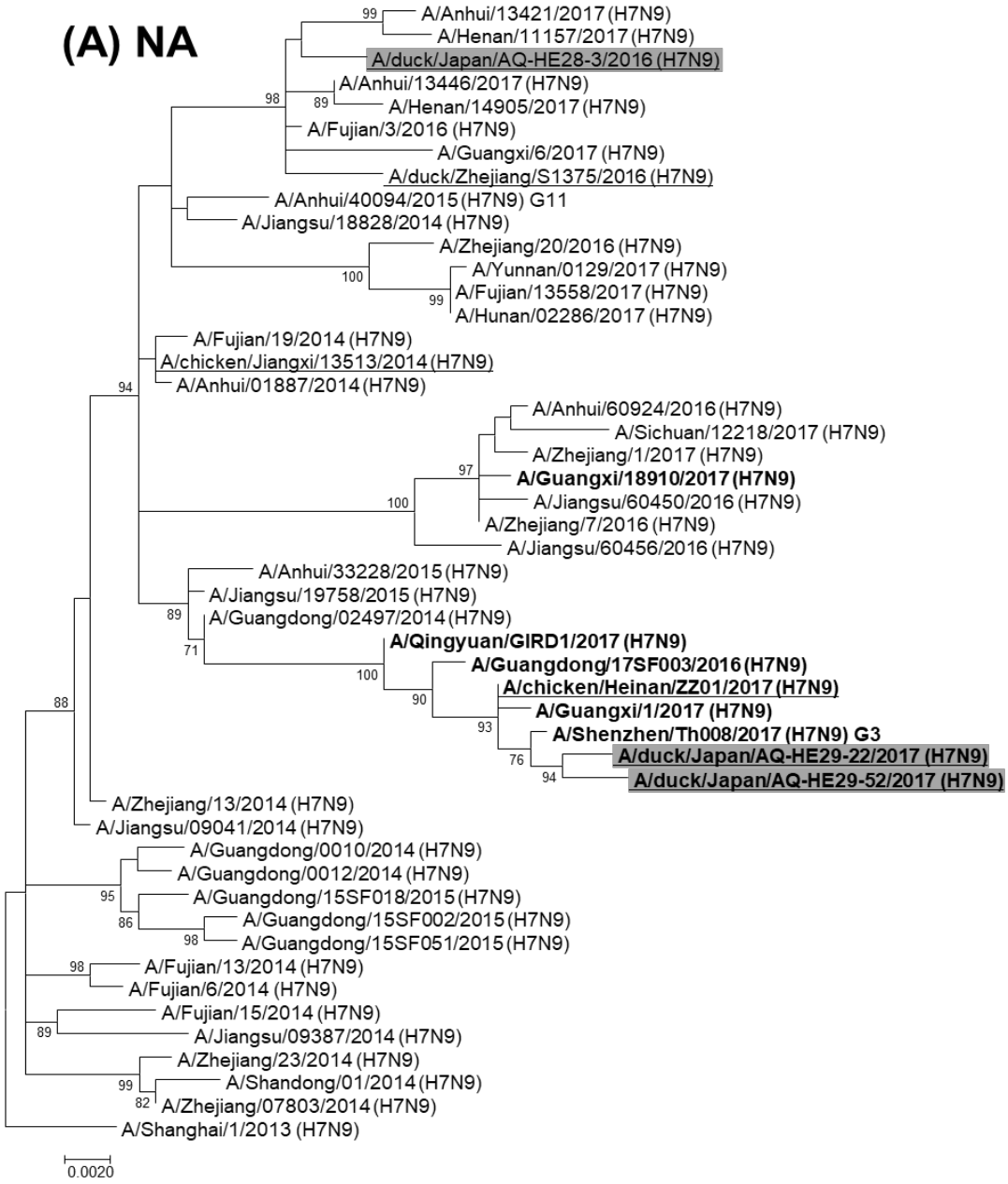
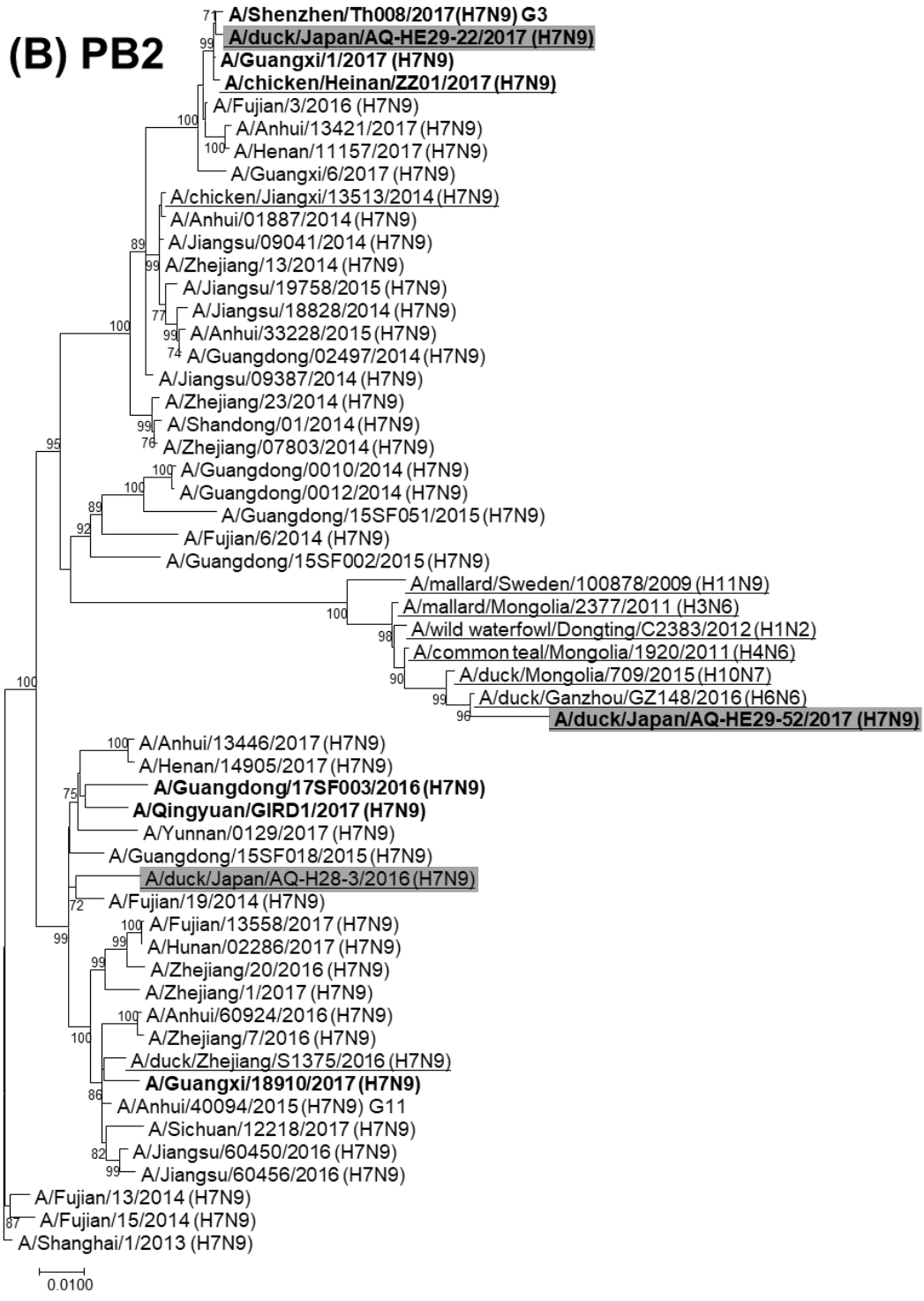



**Figure 6. Phylogenetic tree for the NA (A), PB2 (B), PB1 (C), PA (D), NP (E), M (F), and NS (G) genes of the H7N9 isolated viruses.** The trees were analyzed by the maximum-likelihood method using MEGA 7.0 software (http://www.megasoftware.net/). The horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. Digits at the nodes indicate confidence levels in a bootstrap analysis with 1,000 replications. The viruses isolated in this study are highlighted in gray. The viruses isolated in birds are underlined. Highly pathogenic avian influenza viruses are indicated in bold.


(B) PB2
A/Shenzhen/Th008/2017(H7N9) G3
A/duck/Japan/AQ-HE29-22/2017 (H7N9)
A/Guangxi/1/2017 (H7N9)
A/chicken/Heinan/ZZ01/2017 (H7N9)
A/Fujian/3/2016 (H7N9)
A/Anhui/13421/2017 (H7N9)
A/Henan/11157/2017 (H7N9)
A/Guangxi/6/2017 (H7N9)
A/chicken/Jiangxi/13513/2014 (H7N9)
A/Anhui/01887/2014 (H7N9)
A/Jiangsu/09041/2014 (H7N9)
A/Zhejiang/13/2014 (H7N9)
A/Jiangsu/19758/2015 (H7N9)
A/Jiangsu/18828/2014 (H7N9)
A/Anhui/33228/2015 (H7N9)
A/Guangdong/02497/2014 (H7N9)
A/Jiangsu/09387/2014 (H7N9)
A/Zhejiang/23/2014 (H7N9)
A/Shandong/01/2014 (H7N9)
A/Zhejiang/07803/2014 (H7N9)
A/Guangdong/0010/2014 (H7N9)
A/Guangdong/0012/2014 (H7N9)
A/Guangdong/15SF051/2015 (H7N9)
A/Fujian/6/2014 (H7N9)
A/Guangdong/15SF002/2015 (H7N9)
A/mallard/Sweden/100878/2009 (H11N9)
A/mallard/Mongolia/2377/2011 (H3N6)
A/wild waterfowl/Dongting/C2383/2012 (H1N2)
A/common teal/Mongolia/1920/2011 (H4N6)
A/duck/Mongolia/709/2015 (H10N7)
A/duck/Ganzhou/GZ148/2016 (H6N6)
A/duck/Japan/AQ-HE29-52/2017 (H7N9)
A/Anhui/13446/2017 (H7N9)
A/Henan/14905/2017 (H7N9)
A/Guangdong/17SF003/2016 (H7N9)
A/Qingyuan/GIRD1/2017 (H7N9)
A/Yunnan/0129/2017 (H7N9)
A/Guangdong/15SF018/2015 (H7N9)
A/duck/Japan/AQ-H28-3/2016 (H7N9)
A/Fujian/19/2014 (H7N9)
A/Fujian/13558/2017 (H7N9)
A/Hunan/02286/2017 (H7N9)
A/Zhejiang/20/2016 (H7N9)
A/Zhejiang/1/2017 (H7N9)
A/Anhui/60924/2016 (H7N9)
A/Zhejiang/7/2016 (H7N9)
A/duck/Zhejiang/S1375/2016 (H7N9)
A/Guangxi/18910/2017 (H7N9)
A/Anhui/40094/2015 (H7N9) G11
A/Sichuan/12218/2017 (H7N9)
A/Jiangsu/60450/2016 (H7N9)
A/Jiangsu/60456/2016 (H7N9)
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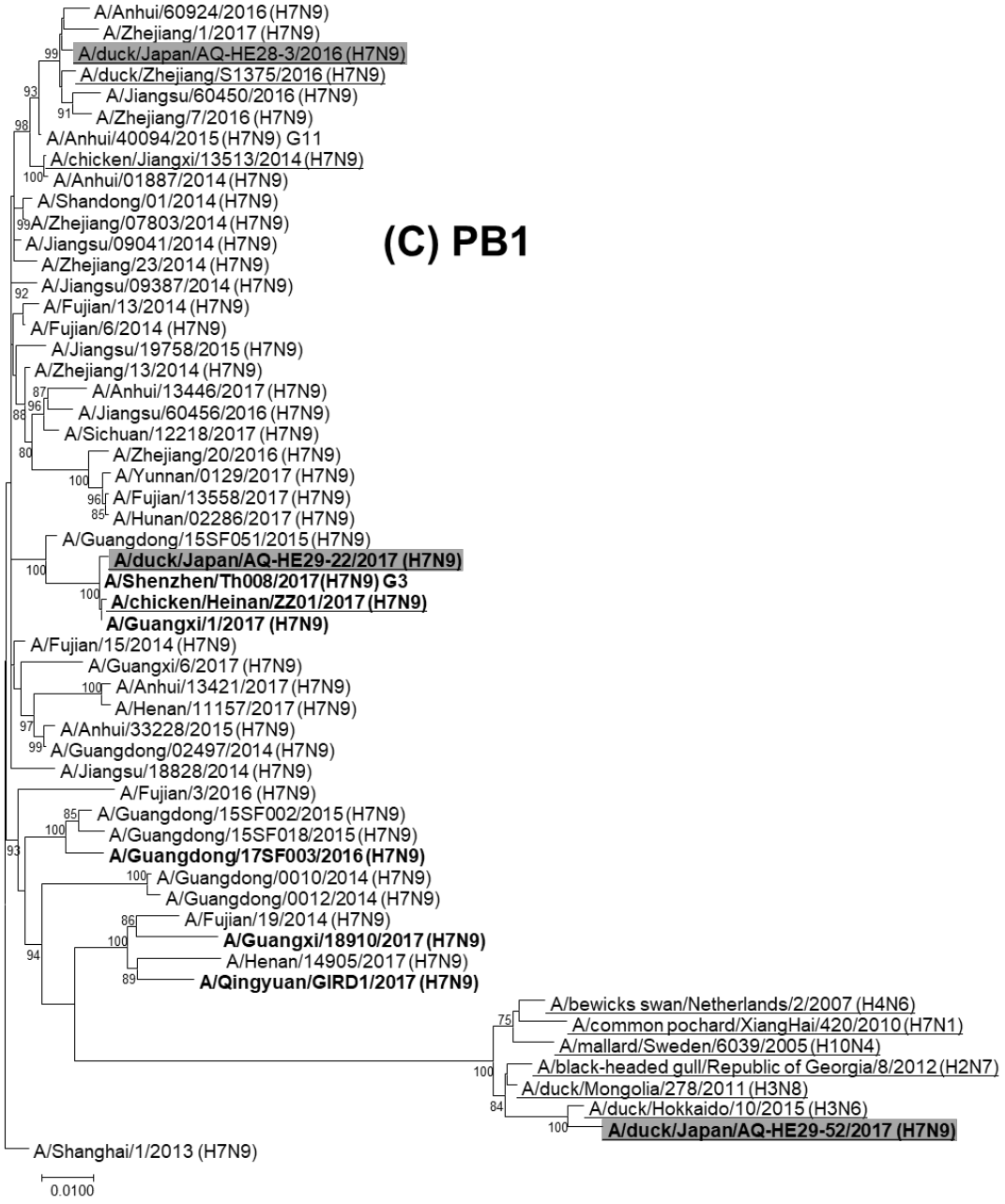
(C) PB1
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A/bewicks swan/Netherlands/2/2007 (H4N6)
A/common pochard/XiangHai/420/2010 (H7N1)
A/mallard/Sweden/6039/2005 (H10N4)
A/black-headed gull/Republic of Georgia/8/2012 (H2N7)
A/duck/Mongolia/278/2011 (H3N8)
A/duck/Hokkaido/10/2015 (H3N6)
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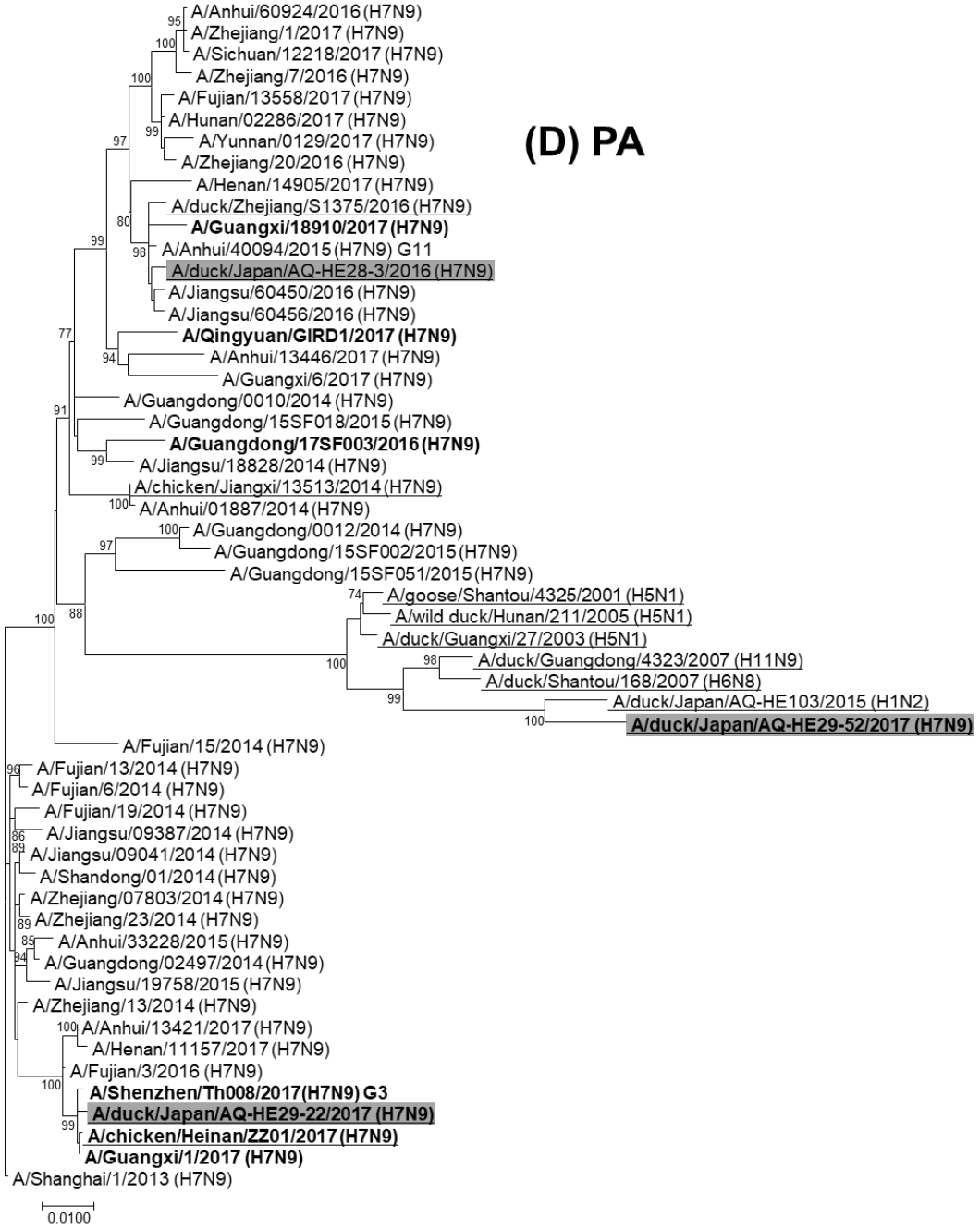
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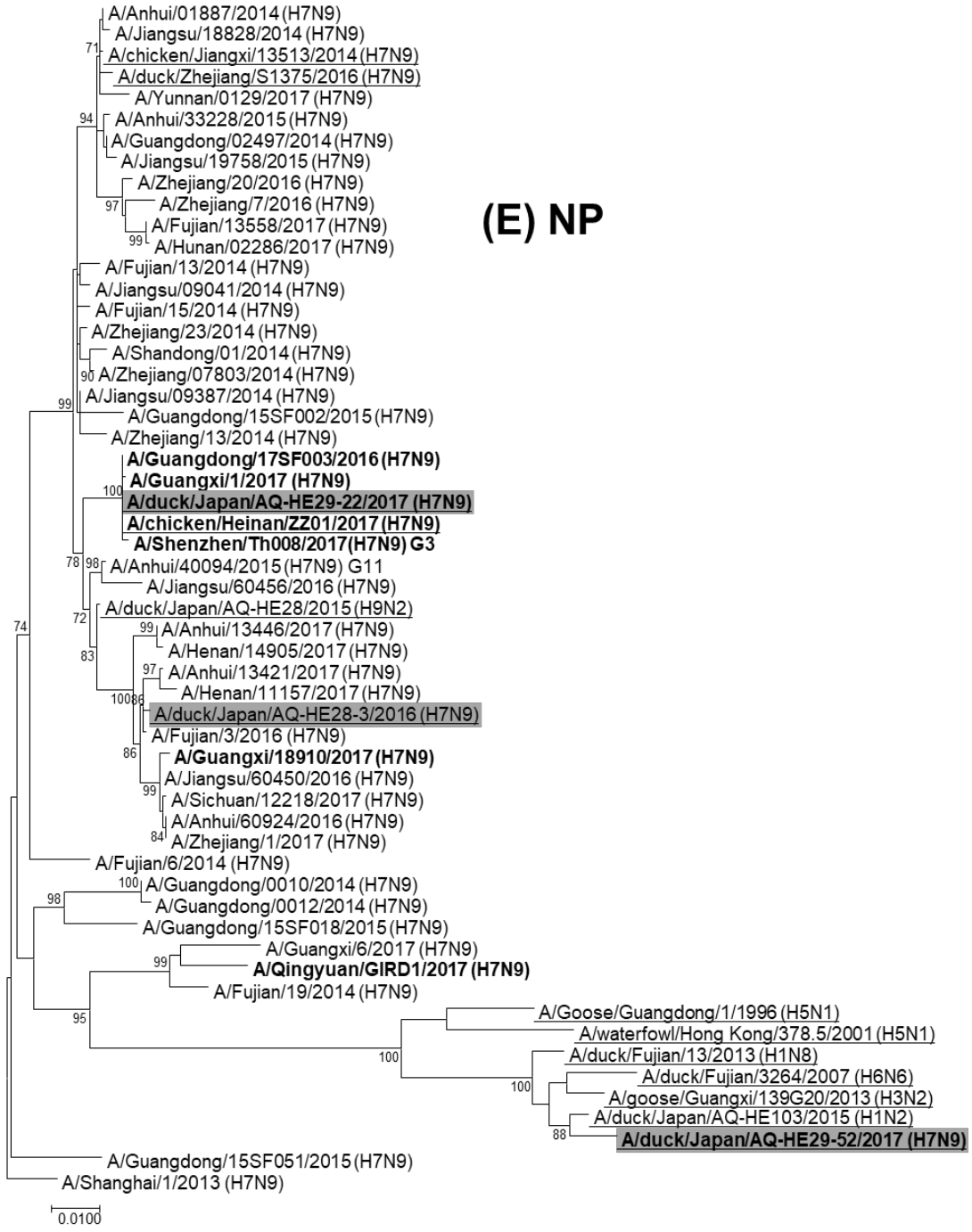
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A/Guangxi/6/2017 (H7N9)
A/Qingyuan/GIRD1/2017 (H7N9)
A/Fujian/19/2014 (H7N9)
A/Goose/Guangdong/1/1996 (H5N1)
A/waterfowl/Hong Kong/378.5/2001 (H5N1)
A/duck/Fujian/13/2013 (H1N8)
A/duck/Fujian/3264/2007 (H6N6)
A/goose/Guangxi/139G20/2013 (H3N2)
A/duck/Japan/AQ-HE103/2015 (H1N2)
A/duck/Japan/AQ-HE29-52/2017 (H7N9)
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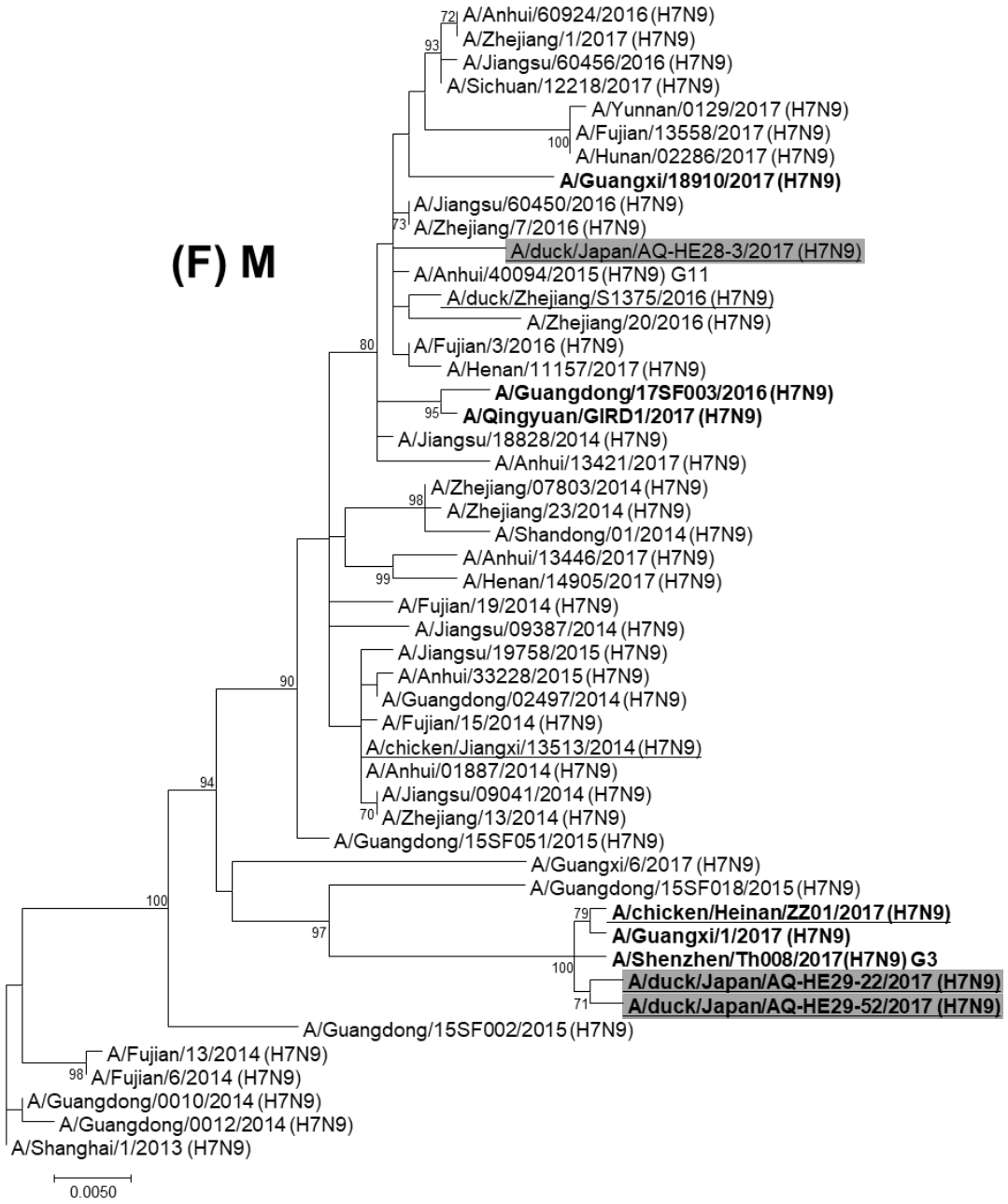
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A/Yunnan/0129/2017 (H7N9)
A/Fujian/13558/2017 (H7N9)
A/Hunan/02286/2017 (H7N9)
A/Guangxi/18910/2017 (H7N9)
A/Jiangsu/60450/2016 (H7N9)
A/Zhejiang/7/2016 (H7N9)
A/duck/Japan/AQ-HE28-3/2017 (H7N9)
A/Anhui/40094/2015 (H7N9) G11
A/duck/Zhejiang/S1375/2016 (H7N9)
A/Zhejiang/20/2016 (H7N9)
A/Fujian/3/2016 (H7N9)
A/Henan/11157/2017 (H7N9)
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A/Qingyuan/GIRD1/2017 (H7N9)
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A/Anhui/13421/2017 (H7N9)
A/Zhejiang/07803/2014 (H7N9)
A/Zhejiang/23/2014 (H7N9)
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A/Guangxi/1/2017 (H7N9)
A/Shenzhen/Th008/2017(H7N9) G3
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A/duck/Japan/AQ-HE29-52/2017 (H7N9)
A/Guangdong/15SF002/2015 (H7N9)
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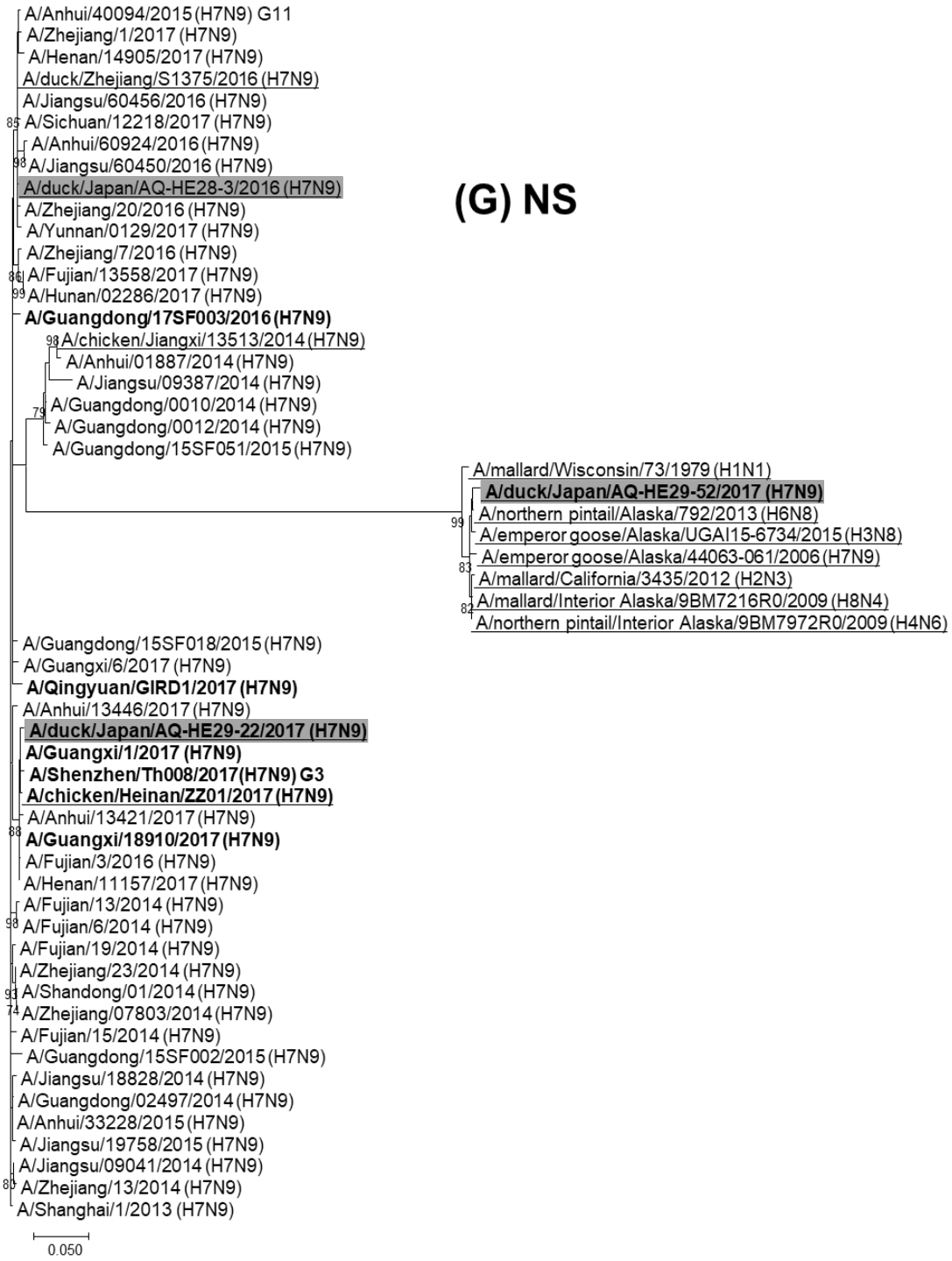
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86
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99
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A/duck/Japan/AQ-HE29-52/2017 (H7N9)
99
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83
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A/mallard/Interior Alaska/9BM7216R0/2009 (H8N4)
82
A/northern pintail/Interior Alaska/9BM7972R0/2009 (H4N6)
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A/Anhui/13421/2017 (H7N9)
88
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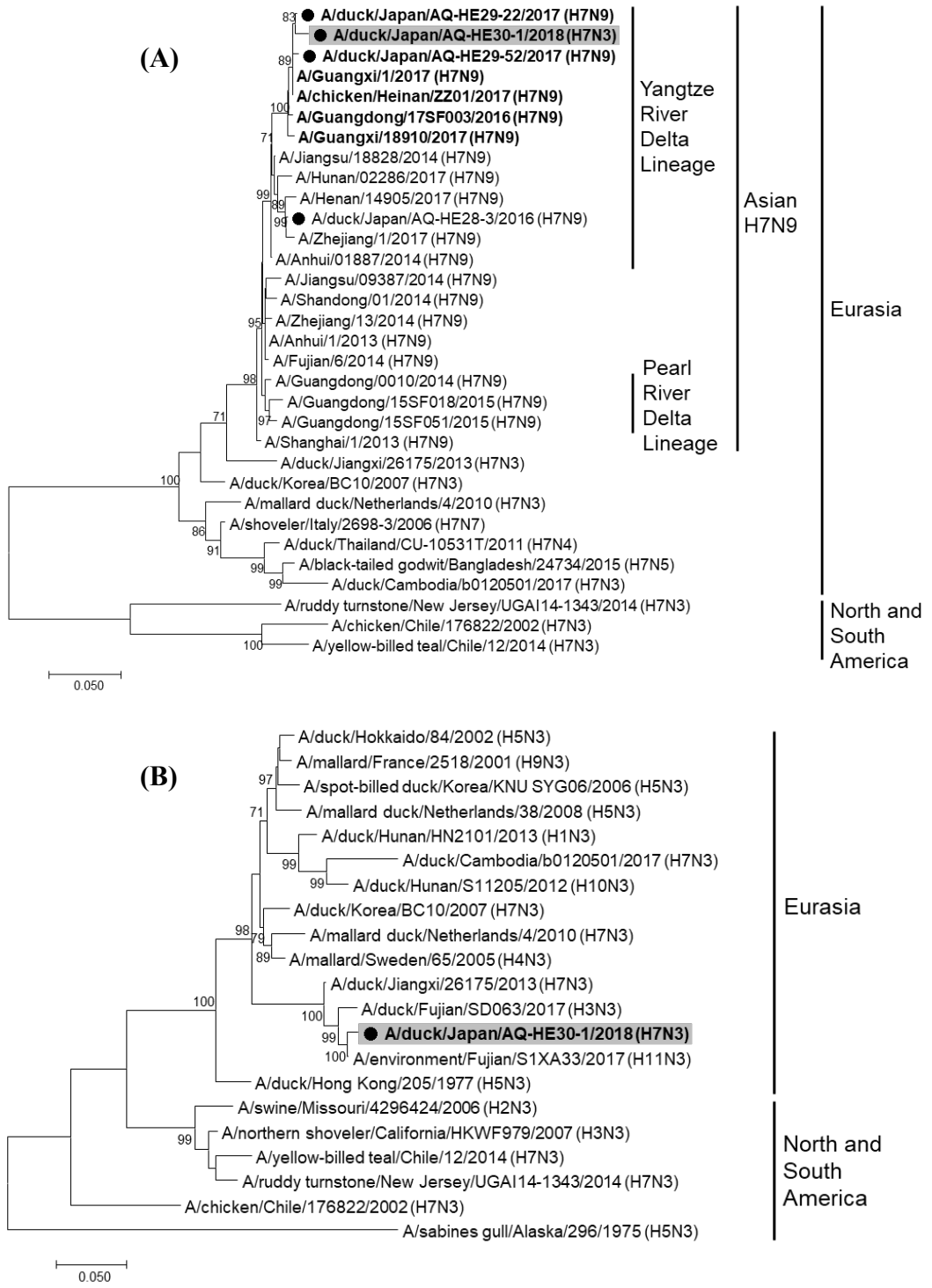


**Figure 7. Phylogenetic analysis of the HA gene of H7 avian influenza virus (A) and the N3 NA gene of avian influenza virus (B):** The nucleotide sequences of the H7 HA and N3 NA genes were analyzed by the maximum-likelihood method with the corresponding genes of reference strains using MEGA 7.0 software (http://www.megasoftware.net/). The horizontal distances in the trees are proportional to the minimum number of nucleotide differences that are required to join nodes and

sequences, and the values at the nodes indicate the confidence levels in the bootstrap analysis with 1,000 replications. The virus that was isolated in this study is highlighted in gray, while highly pathogenic avian influenza viruses are indicated in bold. Viruses that have been isolated from illegally brought meat products are marked with black dots.

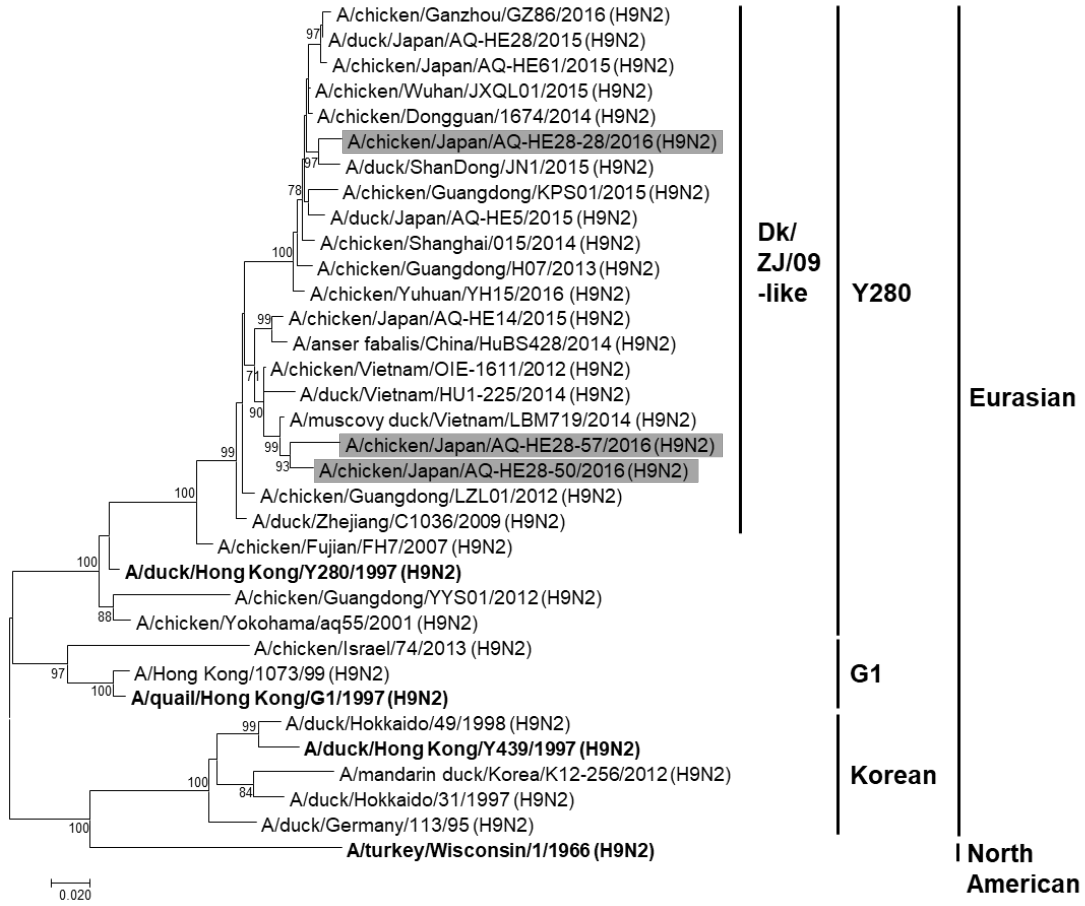


**Figure 8. Phylogenetic tree for the H9 HA genes:** The nucleotide sequences of the H9 HA genes were analyzed by the maximum-likelihood method along with the corresponding genes of reference strains using MEGA 7.0 software and divided into two lineages: Eurasian and North American. Eurasian H9 viruses were clustered into three sublineages: Y280, G1, and Y439. Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. Digits at the nodes indicate confidence levels in a bootstrap analysis with 1,000 replications. Viruses isolated in this study are highlighted in gray, and the representatives of each sublineage are indicated in bold.

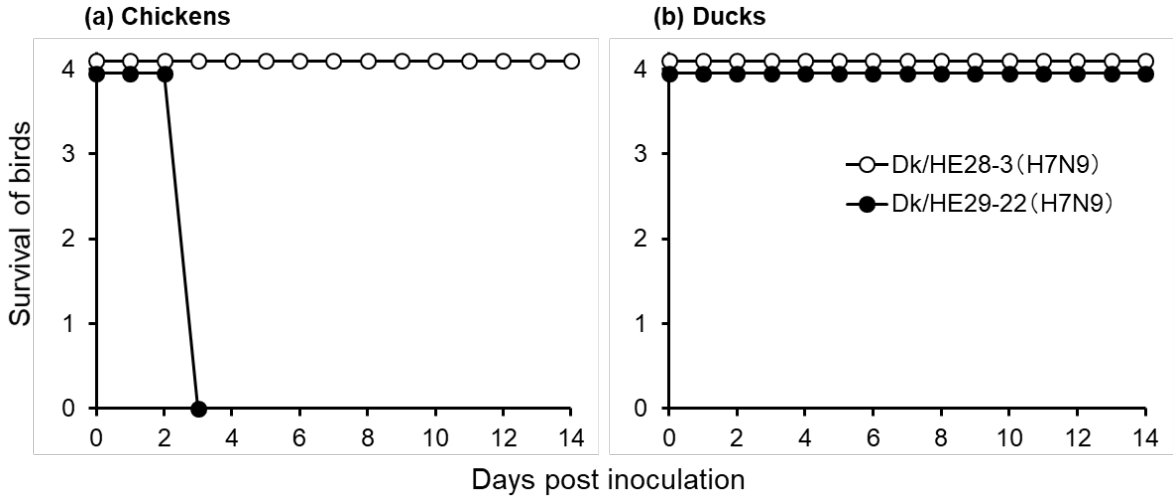


**Figure 9. Survival of chickens (a) and ducks (b) inoculated with each virus:** Four 4-week-old chickens and ducks were inoculated with $10^{6.0}$ $EID_{50}$ of Dk/HE29-22 (H7N9) or Dk/HE28-3 (H7N9). Survival of inoculated chickens and ducks was observed for 14 days after inoculation.

# Conclusion

Avian influenza is highly contagious disease in poultry and wild birds. AIVs cause a mild or asymptomatic infection in their natural reservoir of wild aquatic birds. However, especially H5 and H7 subtypes of viruses they have the potential to mutate into highly pathogenic avian influenza viruses when they are circulating in poultry. In addition to a cause of a great loss to the poultry industry, HPAIVs pose a great threat to the human health since it is capable to infect humans.

In the present study, surveillance of avian influenza virus in poultry products which illegally brought into airports or ports in Japan by passengers and confiscated by baggage inspection was conducted from 2015 to 2018. The HPAIVs of H5N1 and H5N6 subtypes were isolated from poultry products as described in Chapter I, which is the first report of HPAIVs isolated from raw poultry products illegally brought by flight passengers. Genetic analysis revealed that these isolates were closely related to the HPAIV strains reported in the region where the contaminated poultry products were thought to be brought from. The results demonstrate that contaminated poultry products play a role in carrying infectious AIVs including HPAIVs across the border.

In the surveillance from May 2016, the novel H7N9 subtype began to be isolated from poultry products illegally brought from China as described in Chapter II. The H7N9 subtype of LPAIV was isolated earlier than that of HPAIV in accordance with the epidemic situation in China. The present LPAIV, Dk/HE28-3 (H7N9) isolated in 2016 showed more efficient replication in chicken respiratory tissues than A/Anhui/1/2013 (H7N9) which was the ancestor of the novel H7N9. Furthermore, the frequency of virus recovery and GM virus titre in chicken tissues inoculated with HPAIV Dk/HE29-22 (H7N9) isolated in 2017 was even higher than those of chickens inoculated with Dk/HE28-3 (H7N9), indicating that the viral adaptation of novel H7N9 viruses to chickens has progressed steadily in the field.

In addition to the novel H7N9 AIVs, a multiple reassortant H7N3 HPAIV was isolated for the first time from confiscated poultry product brought from China. These continuous detections of AIVs which are capable to infect humans in poultry products indicate the potential risk of infection to human who treat the contaminated products. The number of confiscated cases of illegally brought animal products into Japan has been increasing and reached 110, 000 cases in 2019. In responding to this situation, AQS has strengthened quarantine measures. In order to prevent a recurrence of bringing animal products, AQS has begun to issue written warning to those who illegally brought animal products and created a database of them. When they were deemed to have committed malicious violation of law, AQS shall report to the police; nine people in six

cases have been arrested on suspicion of violating the Act on Domestic Animal Infectious Diseases Control in fiscal year 2019 (https://www.maff.go.jp/aqs/languages/attach/pdf/info-35.pdf). Since July 2020, the maximum possible fine of this law was raised from 1 million yen to 3 million yen. In addition, the number of the quarantine detector dogs also will be increased to 140 by the end of fiscal year 2020.

These results of this study clearly demonstrated that infectious HPAIVs were brought by international flight passengers through contaminated poultry products. These results also help further understanding of the circulation and genetic evolution of AIVs in endemic areas and provide the scientific basis for strengthening border control at the international airports and ports in Japan.

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## Summary in Japanese（和文要旨）

鳥インフルエンザウイルスはA型インフルエンザウイルスに分類され、野鳥や家きんに対して感染性を示す病原体である。カモ類に代表される野生の水きん類は鳥インフルエンザウイルスの自然宿主と考えられており、こうした野生水きん類で循環する鳥インフルエンザウイルスは非病原性であることが知られている。しかし、家きんへの感染を繰り返すことにより、ニワトリに対する高い病原性を獲得することがあり、これまでのところH5亜型及びH7亜型の鳥インフルエンザウイルスにおいて高病原性化することが報告されている。ニワトリに対し高い病原性を示す鳥インフルエンザウイルスは高病原性鳥インフルエンザウイルスと呼ばれ、アジア、ヨーロッパ、アフリカ、北米の家きんや野鳥に拡大し、経済的な被害をもたらしている。鳥インフルエンザウイルスは渡り鳥を介して国境を越えて長距離伝播するほか、感染した家きんが摘発を受けずに畜産物処理され長距離輸送された場合にも、長距離伝播する要因となる。

近年、国際空海港に違法に持ち込まれる畜産物の摘発件数は増加しており、家きん畜産物を介した鳥インフルエンザウイルスの侵入について危惧される。このため、本研究では全国の空海港に携帯品として持ち込まれ、動物検疫所における検査で任意放棄された未加熱の家きん畜産物を対象に鳥インフルエンザウイルス汚染状況の調査を実施した。

2015年～2016年に実施した調査では高病原性のH5亜型を含む8株の鳥インフルエンザウイルスが分離され、分離株は当時中国を始めとする東アジアで流行している株と遺伝学的に近縁であることが分かった。ニワトリに対して致死的な症状を示すH5亜型高病原性株が鶏畜産物から分離されたことは、当該感染鶏が摘発を受けずにと殺され、畜産物処理された事を示している。中国や一部の国で認められる家きんへのワクチン接種は感染鶏の発見を見逃す危険性を伴う。また、H5亜型高病原性株は水きん類であるバリケンの畜産物からも分離されており、接種試験の結果からこれらの株はバリケンに対する病原性は低いことが示唆された。こうした家きんへの感染は高病原性株を拡散させる要因となることから、鳥インフルエンザウイルスの流行とさらなる拡散を防ぐためには、継続的なサーベイランスとワクチンに頼らない摘発・淘汰の実施が望まれる。

2016年～2018年に実施した調査では中国本土以外で家きんから初めてH7N9亜型株が分離され、H7N9亜型のリアソータントウイルスであるH7N3亜型株も初めて分離された。いずれもバリケンの畜産物から分離されており、感染個体が摘発を受けずに畜産物処理されたものと考えられた。ニワトリを用いた接種試験から2016年に分離された低病原性H7N9亜型株Dk/HE28-3 (H7N9)は、2013年にヒトに致死的感染を起こしたH7N9亜型株A/Anhui/1/2013 (H7N9)

と比較し、ニワトリの主要臓器における増殖性は高いことが示唆された。また、2017 年以降に分離された高病原性 H7N9 亜型株 Dk/HE29-22 (H7N9)を用いて実施したニワトリへの接種試験では、ニワトリの主要臓器における増殖性はさらに高くなっており、流行地における H7N9 亜型株のニワトリにおける馴化が進行していることが示唆された。ニワトリに対する病原性においても 2017 年に分離された Dk/HE29-22 (H7N9)の IVPI が 2.88 であったことに対し、2018 年に分離された Dk/HE30-1 (H7N3)の IVPI は 2.99 であったことから、中国 H7 亜型株のニワトリに対する病原性も高まっていることが示唆された。

国際旅客における畜産物の違法持ち込みが後を絶たない状況の中、2019 年 4 月以降に家畜伝染病予防法が改正され、畜産物の違法持ち込みに係る罰則が強化された。畜産物の違法な持ち込みが発覚した場合には、違反者に警告書を発出し、違反した旅客の情報を記録し再発の防止を図るとともに、悪質性を認める場合には警察への通報も行われる。罰則が強化されて以降、2020 年 10 月時点において 6 件 9 名が逮捕されている。さらに、2020 年 7 月以降、違反した際の罰則金の最高額が引き上げられたほか、空港における検疫探知犬の更なる増頭も計画されており、畜産物の違法持ち込みに対する水際検疫の一層の強化が進められている。

本研究により、国際旅客により違法に持ち込まれる家きん畜産物と一緒に感染性のある鳥インフルエンザウイルスが持ち込まれている現状が明らかになった。畜産物を介したウイルスの侵入防止には水際における検疫の強化に加え、持ち込みを避けるための旅客への違法性の周知が重要となる。本研究で得られた成果は、違法に持ち込まれる畜産物が感染性ウイルス拡散の要因となることについての科学的根拠となり、水際検疫の重要性を後押しするものとなる。